Repair of critical bone defects with injectable platelet rich plasma/bone marrow-derived stromal cells composite: experimental study in rabbits

Kritik kemik hasarlarında trombositce zengin plazma/kemik iliği kaynaklı stromal hücrelerin kompozitle enjekte edilerek tamir edilmesi: Tavşanlarda deneysel çalışma

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BACKGROUND

Platelet-rich plasma (PRP) has been applied to promote bone healing and developed as a novel material for bone regeneration. This study aimed to investigate the feasibility of PRP carrier to deliver bone marrow derived stromal cells (BMSCs) and regenerate bone tissues to reconstruct critical bone defects in rabbits.

METHODS

Critical sized defect were made on eighteen rabbits' crania and treated by different composites: BMSCs/PRP (n=6); Autogenous particulate cancellous bone group (n=6) and PRP alone group (n=6). The defects were evaluated by gross observation, radiographic examination, histological examination, and mechanical examination at 12 weeks postoperatively.

RESULTS

The results showed that repair of bone defect was the least in PRP alone group, and significant new bone formation could be observed in BMSCs/PRP group and particulate cancellous bone group, radiopacity area in BMSCs/PRP group attained 76.5%, which was in the same range of that in autogenous particulate cancellous bone group (82.4% in radiopacity area), compressive strength of engineered bone in BMSCs/PRP group attained 71% of that in autogenous particulate cancellous bone group ($p<0.05$).

CONCLUSION

These data implicated that BMSCs delivered from PRP gel can repair bony defect in immunocompetent animals, and the tissue engineered bone in BMSCs/PRP group is comparable to autogenous particulate cancellous bone group for the repair of critical-sized bone defect.

Key Words: Bone marrow-derived stromal cells; bone regeneration; injectable hydrogel; platelet rich plasma; rabbits; tissue engineering/methods.

AMAÇ

Trombositce zengin plazma (TZP), kemik iyileşmesine katkıda bulunmak üzere uygulanmış ve kemik yenilenmesine yönelik yeni bir materyal olarak geliştirilmiştir. Bu çalışmada tavşanlarda, kemik iliği kaynaklı stromal hücreler (KİKSH) vermek ve kritik kemik iliği hasarlarını düzeltmek için kemik dokularını yenilemek üzere TZP taşıyıcılığının fizibilitesinin incelenmesi amaçlandı.

GEREÇ VE YÖNTEM

On sekiz tavşanın kafatasına ciddi ölçüde hasar verildi ve bunlar değişik kompozitlerle tedavi edildi: KİKSH/TZP (n=6); otojen partiküler kansellöz kemik grubu (n=6) ve tek başına TZP grubu (n=6). Hasarlar, ameliyattan sonra 12. haftada gros gözlem, radyografik, histolojik ve mekanik incelemelerle değerlendirildi.

BULGULAR

Kemik hasarı tamirinin tek başına TZP grubunda en az düzeyde olduğunu, KİKSH/TZP grubu ile partiküler kansellöz kemik grubunda anlamlı yeni kemik oluşumu gözlenebileceğini, radyoopasite alanının KİKSH/TZP grubunda otojen partiküler kansellöz kemik grubundakinin (radyoopasite alanında %82.4) %76.5'i oranında kazanıldığını, planlanarak düzenlenmiş kemiğin kompresif gücünün KİKSH/TZP grubunda otojen partiküler kansellöz kemik grubundakinin %71'i oranında kazanıldığını gösterdi ($p<0.05$).

SONUÇ

TZP jelden salıverilen KİKSH'lerin, bağışıklık sistemi normal olan hayvanlarda kemik hasarını tamir edebileceğini ve KİKSH/TZP grubundaki planlanarak düzenlenmiş kemiğin kritik ölçüdeki hasarının tamirinde otojen partiküler kansellöz kemik grubundaki ile karşılaştırılabilir olduğunu göstermiştir.

Anahtar Sözcükler: Kemik iliği kaynaklı stromal hücreler; kemik yenilenmesi; şırınga edilebilir hidrojel; tavşanlar; trombositce zengin plazma; doku mühendisliği/yöntemler.

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Bone regeneration is often needed in orthopaedic or craniofacial surgery for the reconstruction of bone defects caused by trauma or tumor resection. Autologous bone grafting is widely accepted as the gold standard for the treatment of bone defects and non-unions, however, serious drawbacks, such as a prolonged operation time and donor site morbidity in about 10-30% of the cases,[1] sitimulate people to find better approach to repair bony defects.

Advances in tissue engineering during the past decade have enabled this technique to hold promise for the eventual application of guided osseous tissue regeneration. Scaffold, which acted as cell carrier, plays an important role in maintaining cells in defect sites, thus facilitates to the osteogenesis. Both polymeric materials[2] and ceramics have been studied as means to repair defects in bone, among them, porous bioceramics such as hydroxyapatite (HA) and beta-tricalcium phosphate (beta-TCP) represent primary candidate bone substitutes.[3,4] They display excellent osteoconductive properties, can be fabricated into custom-designed shape and size, and are free from risks of rejection or infection. However, an open operation is often needed to implant the cell/scaffold composite, therefore, finding a less invasive way to deliver the complex into the defect site would be desirable. In addition, to treat irregular bone defects resulting from the surgical removal of lesions or traumatic bone loss, cell delivery systems with better plasticity and adhesive fixation into defect site would be more appropriate than solid or block scaffold,[5] especially in the application of craniofacial regions. Therefore, there have been great interests in developing cell carriers which are injectable as well as biodegradable. For this purpose, many injectable materials have been developed for bone tissue engineering. In our previous study,[6] polyethylene oxide hydrogel was used as a vehicle for injecting culture-expanded osteogenic cells to generate new bone, although animal experiments revealed the histological architecture of newly formed bone was similar to that of native bone, the slowly degradation rate and uncontrollable long-term biological behaviors of synthetic hydrogels still impedes their clinical application.[7,8]

Many nature-derived materials such as collagen gel, fibrin glue and chitosan were also developed as injectable carriers for mesenchymal stem cells, which owns better biocompatibility and biodegradability.[9] Furthermore, Spitzer et al. fabricated calcium phosphate particles/fibrin glue carriers, and mixed osteogenic cells in this three-dimensional scaffold,[10] which enabled injectable carriers to keep stable form and resist stress from surgical instruments and *in vivo* compression.

Platelet rich plasma (PRP) is an autologous source of various growth factors, and has been widely used in oral and maxillofacial surgery to promote bone healing.[11-13] From cellular levels, some studies also demonstrated that PRP with certain concentration can improve the proliferation of osteoblast and mesenchymal cells.[14,15] For bone tissue engineering, PRP alone and other scaffolds combined with PRP were also reported.[16] Yamada have used PRP as an autologous scaffold with *in vitro*-expanded mesenchymal stem cells (MSCs) to increase osteogenesis, the results showed the feasibility of PRP as an autologous injectable carrier for bone tissue engineering.[17] However, few studies have evaluated the effect of PRP as injectable scaffold for the reconstruction of critical sized bone defect.

In this study, we have attempted to repair critical calvarial defect in rabbit model, by using PRP as a vehicle for injecting cultured bone marrow derived stromal cells. Good results in this model would indicate a highly effective and less invasive bone graft substitute, which could be useful in many situations of craniofacial bone loss encountered in clinical practice.

MATERIALS AND METHODS

Animal model

Eighteen New Zealand rabbits (3 months of age) were used for this experiment. The operative procedure and the care of the rabbits were performed under the regulation of Experimental Animal Centre, Fourth Military Medical University. The rabbits were acclimated for 1 week before operation and monitored for general appearance, activity, excretion, and weight, then were randomly divided into three groups: experimental group (BMSCs/PRP gel, n=6), positive control group (autogenous particulate cancellous bone, PCB, n=6) and negative control group (PRP gel alone, n=6).

Harvesting and inducing marrow stromal cells in rabbits

Bone marrow stromal cells (BMSCs) in rabbits were harvested and induced as described previously with some modification,[18] the animals were anesthesized before an 11-gauge needle was used to penetrate the cortex of the rabbit iliums, 10 ml of bone marrow was aspirated into a syringe containing 5000 units of heparin. The obtained cells were resuspended and cultured in Dulbecco Modified Eagle Medium (DMEM, Gibco), containing 10% fetal bovine serum (Gibco), ascorbic acid 5 mg/L (Sigma), L-glutamine 0.272 g/L (Sigma). When the cells covered about 70-80% of the bottom of the dishes, induction medium containing ascorbic acid 50 mg/L, dexamethasone 10 nmol/L (Sigma), β-glycerophosphate sodium 10 mmol/L (Sigma), recombinant human bone morphogenetic protein 2100 μg/L (rhBMP-2, Department of biological chemistry, FMMU, China) was applied to improve the osteoblastic phenotype of the marrow stromal cells (Fig. 1a). Before they reached the confluent monolayer, the bone marrow derived osteoblasts were digested by using trypsin 0.25% (Sigma) digestion and harvested by centrifugation. The cell density was adjusted to 5.0 x 10^7/mL with medium before cell seeding.

Preparation of platelet rich plasma and BMSCs/ PRP gel composite

In experimental group, before injecting BMSCs/ PRP gel composite into the body of the animal, 10 mL venous blood was drawn from each of the cell donor rabbits, then processed as reported previously.[19] Briefly, in a 20 mL sterilized syringe containing 1.5 mL of sodium citrate (Sigma, USA). Following above procedures, the blood was subjected to centrifugation for 10 min at 1800 rpm and the obtained supernatant was transferred to another tube. The supernatant was subjected to centrifugation for 10 min at 3600 rpm to obtain platelet poor plasma (PPP) and platelet rich plasma (PRP). The top layer, which consisted of the PPP, was aspirated and put into a new tube. Then about 1.0 mL of PRP was aspirated and put into another tube for cell seeding.

Injection of BMSCs/PRP gel composite

After cultured BMSCs reached confluent monolayer (Fig. 1a), they were collected by trypsin digestion and were centrifuged at 1000 rpm for 5 min, the resulting cell pellet was washed 2 times with PBS to remove residual serum, then cells were counted and resuspended with PRP liquid from the same donor animal, and the cell density was adjusted at 5.0 x 10^7/mL. To activate the PRP, 0.15 mL of $CaCl_2$ and bovine thrombin (Gibco, 1000 U/ml in 100 mg/ml calcium chloride) mixture was added to BMSCs/PRP suspension. To investigate the cell distribution of BMSCs/PRP composite, part of composite was processed for scanning electron microscopy (SEM) examination.

Surgical procedure

New Zealand rabbits were anesthetized with ketamine (50 mg/kg IM) and xylasine (5 mg/kg IM). After disinfecting the operative site, a tongue-shaped incision over the scalp was made through the subcutaneous tissue covering the cranium. The periosteum was resected, and a 15 mm diameter defect was created carefully by dental bar cooled with sterile saline. As Fig. 2 showed, the craniotomy segment was removed and the damage to the

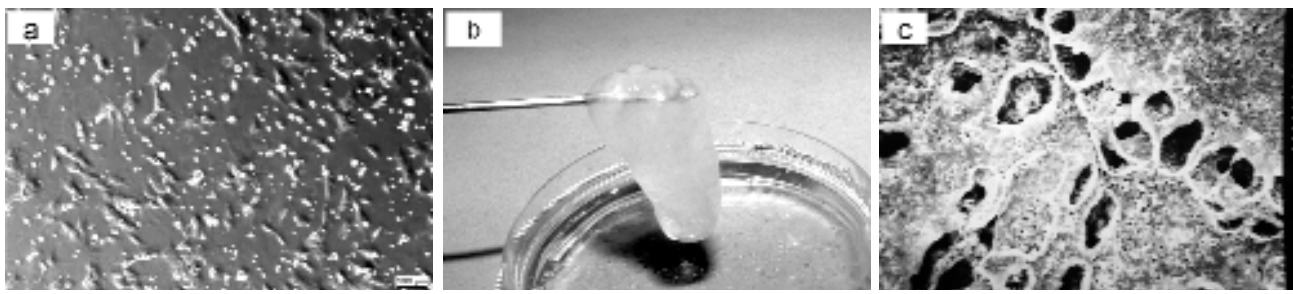

Fig. 1. **(a)** Prior to seeding, phase contrast photomicrograph of BMSC in monolayer culture after induction (bar scale: 100 μm). **(b)** After mixing of PRP and bovine thrombin, the PRP gel was formed after being extruded from syringe. **(c)** Scanning electron microscopic (SEM) observation of complexes prior to implantation. Cells distributed evenly into the hydrogel, (bar scale: 150 μm).

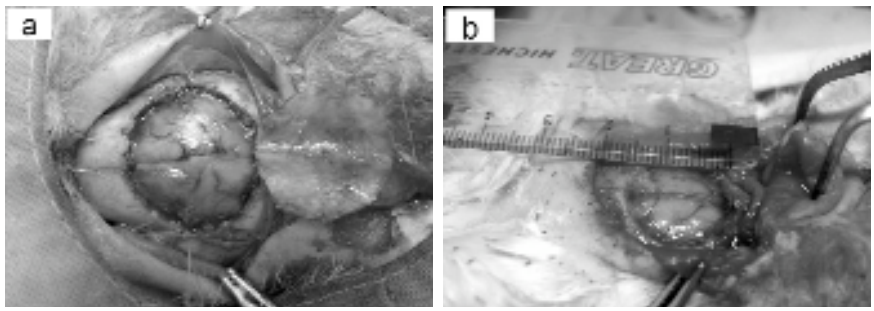

Fig. 2. Photographs of the surgical procedure. **(a)** A circular craniotomy segment was removed and the damage to the underlying dura and superior sagittal sinus vein was avoided. **(b)** Bony defect with 15 mm diameter was created.

underlying dura and superior sagittal sinus vein were avoided. The defect was rinsed with sterile saline thoroughly and bone fragments were washed out. According to the grouping protocol, the animals in experimental group underwent injection of autologous BMSCs/PRP gel composite through a 16 gauge needle (Fig. 1b), SEM images also verified that BMSCs distributed evenly in formed gel (Fig. 1c). Six animals in positive control group were given autogenous particulate cancellous bone (PCB) obtained from iliums, six animals in negative control group were given PRP gel only. Then the scalp was closed with sutures.

Gross observation, radiographic examination

Animals were sacrificed at 12 weeks postoperatively, six in each group, and the whole crania including defect and surrounding bone was dissected with a bone cutter. The specimens in different groups were examined by gross specimen inspection. Radiographs were then taken for the excised cranium to visualize mineralization in the treated defects and the controls. Mineralization in each group was calculated according to qualitative analysis of radiopacity area.

Sample preparation, histological examination

The crania was halved longitudinally in the median plane with use of a diamond saw. One-half of the specimen was fixed in 10% phosphate-buffered formalin and embedded in paraffin wax after decalcification then these specimens were embedded in paraffin and sectioned for hematoxylin & eosin and Masson's trichrome staining. All the histological examinations were done by light microscope. The other half was treated for mechanical test.

Compressive test

The test was performed in an Instron universal testing machine measuring specimens to 50% deformation with a flat-ended 10 cm^2 probe at a constant cross-speed of 1mm/min, after harvesting of the specimens.

Statistical analysis

All the data were analyzed by a Student's t-test with a minimum confidence level of 0.05 for statistical significance. All values were reported as the mean and standard deviation of the mean.

RESULTS

Gross inspection

All the animals survived the intervention and concluded the study. No sign of inflammation or adverse tissue reaction was observed around implants. Gross examination showed that the wound was well healed. Representative gross specimens of each group were shown in Fig. 3. From the gross specimens resected from rabbit models in experimental group (BMSCs/PRP gel) and positive control group, observations and palpation showed that newly formed bone had repaired the defect and restored the continuity of the cranial bone, tissue filling each defect in the cranial bone was hard and non-compressible (Fig. 3a, Fig. 3b). In addition, in group (BMSCs/PRP gel), tissue engineered bone was observed to completely bridge the defect and was indistinguishable at the margins from native bone. In contrast, only a thin fibrous bridge was observed on the top of the defect in PRP gel alone group, no bony tissue filling was observed in defect site (Fig. 3c).

Radiographic examination

Representative radiographic films of each group at 12 weeks postoperatively were shown in Fig. 4. Radiographs of the defect site showed almost complete union in the positive control at the time of har-

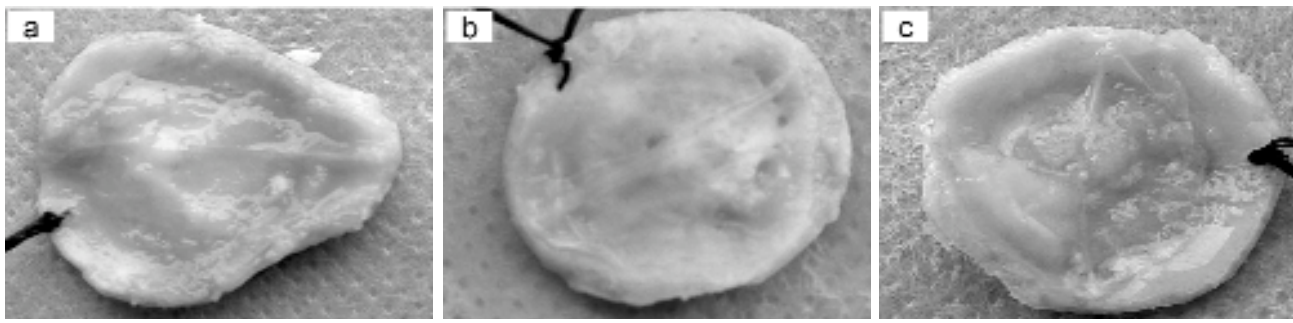

Fig. 3. Gross inspection of rabbit crania 12 weeks after operation, 15 mm critical-sized defects with **(a)** autologous particulate cancellous group, **(b)** BMSCs/PRP group, **(c)** PRP alone group.

vesting. In BMSCs/PRP gel group, the same image could be seen as what was in the positive control group, but transparent image was still seen some place at the edges of the defect (Fig. 4a, Fig. 4b). Only a light radiopacity was noted throughout the whole defect when only PRP gel was implanted (Fig. 4c).

After quantified, the result was presented in Fig. 4d, the radiopacity area occupied most of the defect area (82.4±2.9%) in PCB group, the radiopacity area in BMSCs/PRP gel group occupied 76.5±3.6% of defect area, both groups were in the same range, In contrast, radiopacity area in PRP gel alone group only occupied 15.8%±2.2% of defect area. With

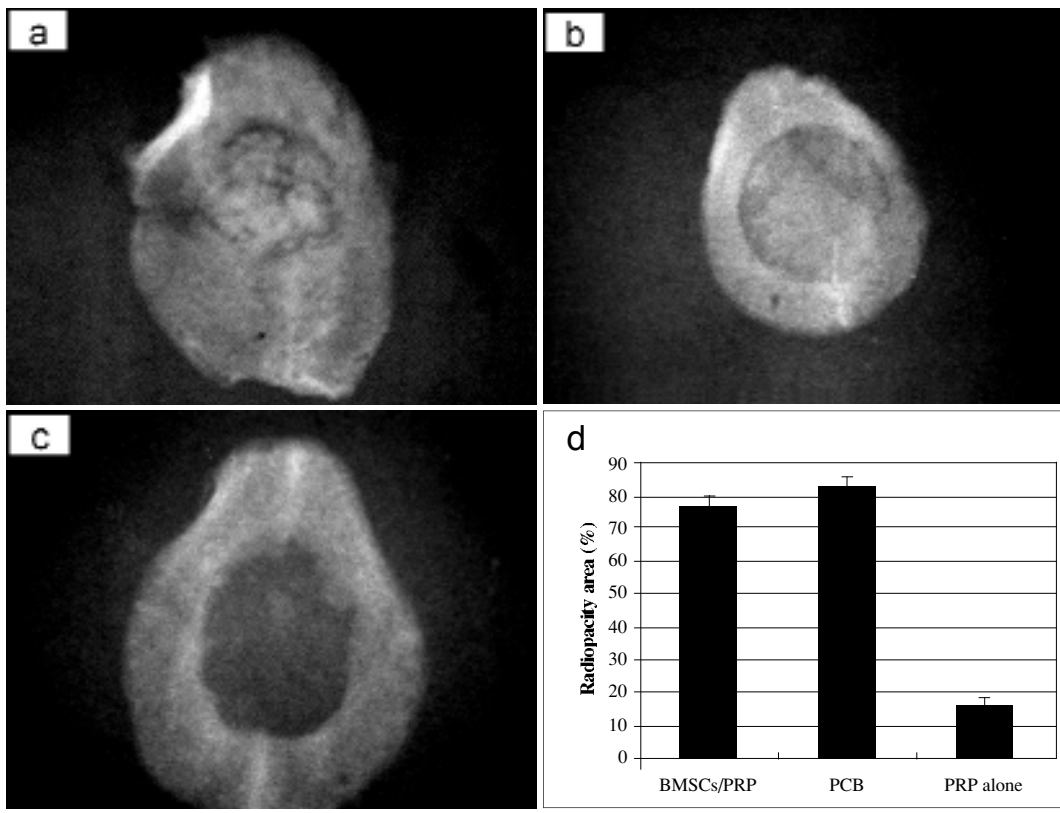

Fig. 4. Radiopacity on cranial defect in different group. **(a)** BMSCs/PRP group. **(b)** PCB group. **(c)** PRP alone group. **(d)** Comparison of radiopacity on cranial defect in different group at 12 weeks. Radiopacity in all the positive control group, MSCs/PRP group were significantly higher than PRP alone group ($p<0.05$). And there was no significant difference between PCB group and BMSCs/PRP group.

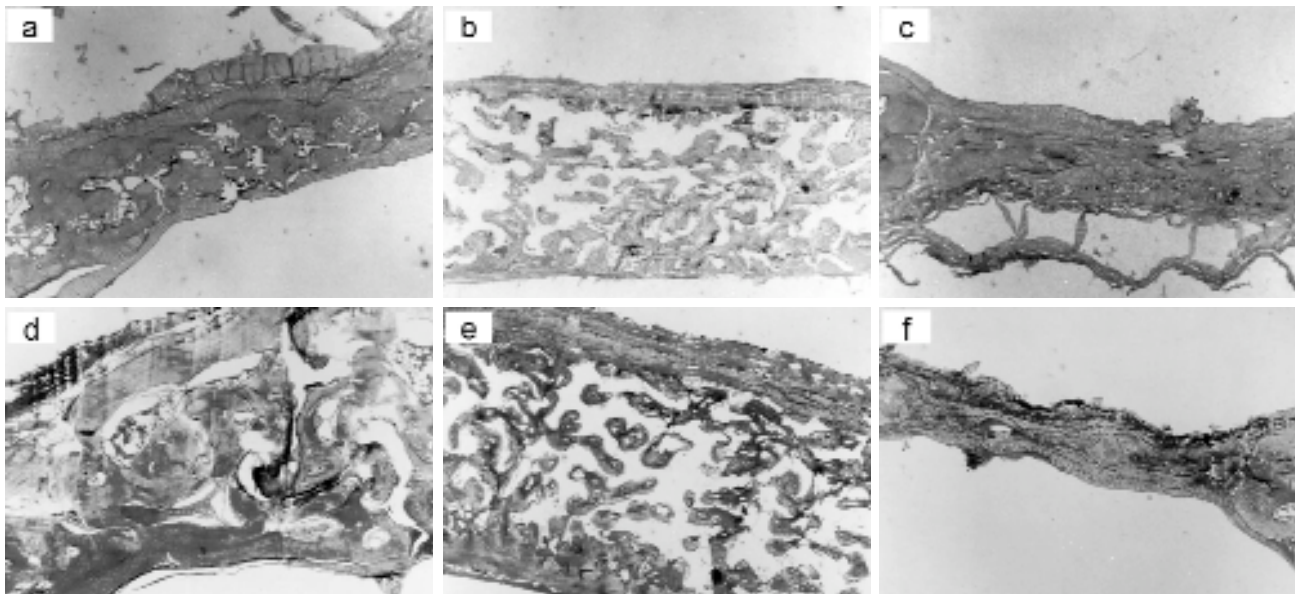

Fig. 5. The histological appearance of critical cranial defects at 12 weeks postoperatively. **(a)** The bone defect site has been fully closed in the BMSCs/PRP treated defect small vessels were observed to penetrated into the formed bone tissues (arrow), H&E x4. **(b)** Bone defect site treated with autologous bone. An extensive network of newly formed bone trabeculae is seen adjacent to the original cortical bone H&E x4. **(c)** Only fibrous tissues can be observed in the defect site H&E x4. **(d)** Masson's trichrome showed massive formation of mature bone-like tissues in the defect of BMSCs/PRP group, as arrows indicate (x10), which is also showed in E group (autologous particulate cancellous bone group, x10)). **(f)** In PRP alone group, Masson's trichrome showed less new bone formation at the surface of the specimen, The rest of the defect is filled with fibrous tissue (x4).

respect to the applied p-level (0.05), no difference between engineered bone and native bone graft was observed ($p<0.05$).

Histological examination

At twelve weeks after operation, in defects filled with BMSCs/PRP gel constructs, an endochondral ossification pattern was observed. Histological analysis with H&E and Masson's trichrome staining revealed abundant bone formed in the healed defects. New bone was mature with histological appearance of trabecular bone throughout the composite, crosslinked osteoid tissue formed replaced the PRP gel, and small diameter blood vessels had penetrated into the bone graft. The development of the bone trabecular and medullary cavity filled with bone marrow were also observed in newly formed bone (Fig. 5a and Fig. 5d). Fig. 5b and Fig. 5e revealed the substantial new bone formation occurred at the defect site in autologous particulate cancellous bone group. New woven bone was observed in bone graft and Masson's trichrome verified the calcification within transplant. In contrast, the group of PRP gel without cells showed large part of defects was filled with fibrous tissues (Fig. 5c), no obvious osteoid synthesis was noticed (Fig. 5f).

Mechanical test

The mean compressive strength in BMSCs/PRP gel group, PCB group and native cranium was 41.6±3.7 N (n=6), 58.5±3.5 N (n=6) and 64.7±1.8 N respectively, with respect to the applied p-level (0.05), the difference between engineered bone and native bone or PCB graft was significant, and the compressive strength in tissue engineered bone reached 71% of that in PCB group (Fig 6).

DISCUSSION

This study further verified that critical bony defects could be reconstructed by using BMSCs/scaffold complex. As an autologous injectable hydrogel, PRP gel provided a 3-D environment for cultured BMSCs, and can fix seeded cells in injected site. Furthermore, injectable PRP gel owns minimally invasive property, better nutrition perfusion and minimally immunological reaction *in vivo*. The results of this experiment demonstrate PRP gel can

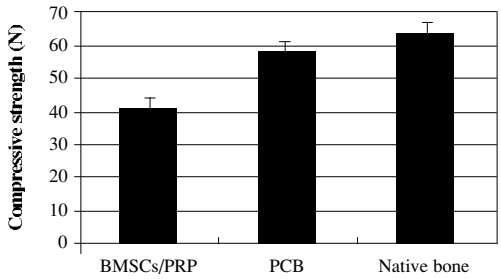

Fig. 6. Compression strength of bone tissues in BMSCs/PRP group, PCB group and native bone group, data was presented as Mean ± SD for all groups (n=6).

be used as a promising injectable scaffold for the reconstruction of critical sized bone defect.

Defects of a size that will not heal during the lifetime of the animal may be termed defects of critical size. The investigation on new bone formation has always been suggested in rabbit critical-sized cranial defects first. In most studies the critical size in rabbit cranium is 15 mm in diameter.[20] In Clokie's study, bone regeneration of commercially available bone substitutes was evaluated in critical sized cranial vault defects (15 mm in diameter) in New Zealand white rabbits.[21] Dean et al.[22] had also investigated the osseointegration of four types of bone graft scaffolds in 15 mm diameter critical-sized rabbit cranial defect and suggested poly (propylene fumarate) as a biocompatible material that provided both a structural and osteogenic substitute for the repair of cranial defects.

An important advantage of tissue engineering is the availability of bone tissue without donor site morbidity. Mesenchymal stem cells are multipotential cells which can be induced to differentiate into bone, cartilage, fat and connective tissue, Mesenchymal stem cells can be aspirated from bone marrow and expanded *in vitro* to a large number,[23] thus provided an ideal source for autologous bone tissue engineering. In previous study, we have observed bone formation using culture-expanded marrow derived osteogenic cells was through endochondral bone formation.[24] As compared with two other kinds of osteogenic cells, osteoblast and periosteal cells, bone marrow derived osteoblasts could be easily obtained by needle aspiration, which avoids traumatic harvesting operations. In present study, represented by percentage of radiopacity area, BMSCs/PRP gel showed good osteogenic capacity which produced similar amount of bone tissues as compared with autologous bone graft. This result showed that BMSCs can efficiently improve the healing capacity of bone when implanted in defect site.

Uniform cell distribution and good nutrition metabolism are essential for tissue formation in the scaffold. However, due to the uncontrollable cell-biomaterial interaction and limited cell seeding efficiency, cell seeding onto porous membranes or solid scaffolds typically does not result in an even cell distribution.[9] In present study, for even distribution, cells were suspended in PRP solutions that subsequently gel, which was similar to fibrin, agarose and alginate that are used frequently as embedding gels in tissue engineering applications.[25,26] Histologically, osteoid tissues formed in BMSCs/PRP gel group were distributed more evenly than that in PCB group. The newly formed cancellous bone was throughout the whole tissues and was infiltrated with blood vessels with approximate diameters. Angiogenesis is essential for the delivery of oxygen and nutrients required for bone formation. Osteoblast-like cells need a supply of oxygen and nutrients from surrounding blood vessels or tissue fluid *in vivo*, the cells inside the porous materials can not obtain a supply of oxygen and nutrients during the culture *in vivo*, this fact remains a difficult problem in tissue engineering when repair large tissue defect. In present study, owning to the hydrogel property of the PRP gel, the fibrin network allows the better perfusion of the nutrition, which supported the viability of osteoblasts before ingrowth of blood vessels. Histological examination showed osteoid tissues formed throughout the defect site, no central necrosis or empty region was noticed in formed tissues. In addition, at 12 weeks postoperatively, an even distributed blood vessels or medullary channels have been reestablished in formed tissues, which was similar to the results observed in PCB group.

For bone defects caused by trauma, irregular shapes were often needed to be restored by bone transplantation. However, the shaping process and fixation of bone grafts were always complicated. As compared with solid or block scaffold, better plasticity and viscidity of hydrogels gelling in situ make them more appropriate to repair bony defect with irregular shapes.[27] In our study, although circular defect can be reconstructed well by using par-

ticulate cancellous bone, distributing particulate cancellous bone evenly and maintaining their sites were more complicated than that in injecting PRP suspended with BMSCs. Histologically, bony tissues formed in BMSCs/PRP gel group was continuous and integrated with native cancellous bone closely, which further verified the superiorities of PRP gel as cell carrier.

Platelet rich plasma (PRP) is derived from autologous blood and provides autologous source of various growth factors such as TGF-β, FGF. However, the *in vivo* effect of PRP on bone healing and bone regeneration was still controversial.[28,29] Tara L et al. evaluate the effect of platelet-rich plasma (PRP) on bone healing, results showed PRP gel alone can not significantly improve the healing of noncritical sized defects in the rabbit cranial model.[30] Our results was in consistent with previous conclusions, bone defects in PRP gel alone groups failed to be repaired by newly formed osteoid tissues. In contrast, more and more *in vitro* studies demonstrated that PRP have favorable effect on human osteoblast-like cells and stromal stem cells,[31] and cell/PRP composite have been approved to be an effective substitute for bone reconstruction.[17] Yamada's experiment has approved that PRP gel combined with MSCs could form mature bone tissues *in vivo* and could be used to repair periodontal bone defect. In these studies, bone defects is limited in size. Our experiment further approved that BMSCs/PRP can be used to reconstructed critical bony defect in cranial bone, which further supported the views that PRP can be used as an autologous bioactive scaffold for tissue engineering.

Despite successful reconstruction of critical calvarial defect in this experiment, it may be argued that poor mechanical stability of PRP gel may limit their load-bearing application in the reconstruction of bony defects. In addition, mechanical test in our experiment also showed that compressive force in bone tissues of BMSCs/PRP group only attained 71% of that in PCB group. Therefore, more suitable tissue engineered construct may be fabricated by combining delivery materials with different properties. In further study, macroporous bioceramic scaffold with predetermined shape would be prefabricated and combined with PRP/BMSCs for the construction of bone tissues in special shape or in load-bearing sites.

In conclusion, this study has shown the successful reconstruction of critical calvarial defect by using PRP seeded with BMSCs. The macroscopic and microscopic results showed the bone tissues regenerated by this injectable bone graft was similar to that in autologous bone graft. These results demonstrated that BMSCs/PRP composite can be used as a novel injectable bone substitute, and provided a valuable alternative to reconstruct critical bony defect in craniofacial regions.

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