Injectable tissue-engineered bone composed of human adipose-derived stromal cells and platelet-rich plasma

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ABSTRACT

This study aimed to evaluate the effects of human platelet-rich plasma (hPRP) on the proliferation and osteogenic differentiation of human adipose-derived stromal cells (hADSCs) and to construct a novel injectable tissue-engineered bone (ITB) composed of hPRP and hADSCs. hADSCs were isolated from liposuction tissues of healthy patients. hPRP was obtained by traditional two-step centrifugation. MTT, alkaline phosphatase (ALP) activity and mineralization assays were used to evaluate the effects of different concentrations of hPRP on cell proliferation and osteogenic differentiation *in vitro*. hADSCs cultured in optimal concentration of activated hPRP were subcutaneously injected into the inguinal groove of nude mice with hPRP and thrombin. X-ray, H&E staining and immunohistochemical analysis were used to examine the bone formation. Studies *in vitro* revealed that cell proliferation, ALP activity and mineralization were induced by hPRP and 10–12.5% of hPRP seemed to be the optimal concentration. Studies *in vivo* showed that this ITB formed bone structure in heterotopic site of nude mice. These findings indicate that the ITB composed of hPRP and hADSCs may represent a prologue for the development of a novel biological solution to bone defect. However, further investigations should be done to fully reveal the characteristics of this ITB.

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1. Introduction

Tissue engineering approach is a promising way to restore bone defect. This strategy consists of three necessary elements [1]: (1) seed cells with a high osteogenic potential, (2) osteogenic growth factors and (3) a three-dimensional scaffold which gives the construct sufficient mechanical properties for loading and facilitates vascularization. For the ideal construct, the seed cells should be autologous and easy to obtain with minimal donor site morbidity [2,3]; the osteogenic growth factor should be easily produced with very low cost and poor immunogenicity [4,5]; and the scaffold should be biodegradable and derived from homologous materials [5,6]. However, recent tissue engineered bone systems still couldn't meet these three requirements at the same time.

To meet these three requirements simultaneously, a novel injectable tissue-engineered bone (ITB) was established in this study. It was composed of three elements: (1) human adipose-derived stromal cells (hADSCs) as seed cells that can be easily obtained with large quantities and least donor site morbidity, which facilitates its autologous application; (2) human platelet-rich plasma (hPRP) released growth factors as osteogenic regulating factors, which is easy to obtain from autologous blood and contains high concentration of platelet-derived growth factor AB (PDGF-AB), transforming growth factorβ1 (TGF-β1), insulin-like growth factor (IGF), and so on [7–9]; and (3) fibrillar three-dimensional scaffold formed by fibrinogen from activated hPRP which is easy to biodegrade and safe for its autologous application.

If this construct can form bone structure *in vivo*, it will represent an ideal tissue-engineered bone which is easily acceptable by patients and doctors for its autologous origin, safety, relatively low cost, and easy obtainability. Meanwhile, it is a novel sample in accordance with the idea of "biological solutions to biological and medical problems" which means using optimized autologously originated biological materials instead of artificial substitutes in the treatment of a wide range of diseases and is now emerging as a new paradigm in different medical fields including bone tissue engineering [4,10]. Moreover, the liquid hPRP and hADSCs could be applied by injection and grafted in minimally invasive way. If this ITB can be applied in the clinic, a novel biological remedy for bone defects will come into being.

To comprehensively evaluate the characteristics of the ITB, the effects of hPRP on the proliferation and osteogenic differentiation of hADSCs were explored in the *in vitro* part of this study, and the morphology and applicability of the ITB were tested through scanning electron microscopy (SEM) and *in vivo* experiments.

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2. Materials and methods

2.1. Materials

All materials were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA).

2.2. Cell isolation and culture

Human adipose-derived stromal cells (hADSCs) were obtained according to previously published methods [11,12]. Briefly, human adipose tissues were obtained with informed consents from five healthy patients who were under liposuction surgery for esthetic reason in the plastic surgery hospital affiliated to Chinese Academy of Medical Science. The study was approved by the Ethics Committee of the Peking University Health Science Center, Beijing, China. The liposuction tissue was washed at least three times with equal volumes of phosphate-buffered saline (PBS) and then digested with 0.075% type I collagenase for 60 min at 37 °C with intermittent shaking. The floating adipocytes were separated from the stromal cells by centrifugal force (300g) for 10 min and the cell pellet was obtained. Then the pellet was resuspended in 160 mM NH_4Cl and incubated at room temperature (RT) for 10 min to lyse contaminating red blood cells. The stromal cells were collected by centrifugation, filtered through a 100-μm nylon mesh to remove cellular debris and were cultured in fresh DMEM containing 10% (v/v) FBS, 100 U/mL penicillin G and 100 μg/mL streptomycin at 37 °C in an incubator with an atmosphere consisting of 95% air, 5% CO_2 and 100% relative humidity. Cells of the third passage were used for the in vitro experiments and all in vitro experiments were repeated three times using hADSCs from the three patients, respectively. Cells of the fourth passage from other two patients were used for the in vivo experiments.

2.3. Preparation of activated platelet-rich plasma

Two whole blood samples (400 mL of each) of healthy adults were obtained from Red Cross Blood Bank of Beijing. The anticoagulant used was citrate-phosphate-dextrose (CPD) and the samples were stored for less than 2 days. hPRP was prepared by a traditional two-step centrifugation procedure [13]. Briefly, whole blood was initially centrifuged at 220g for 15 min. The superstratum of yellow plasma (containing the platelets) was moved into other monovette with a long cannula to exclude large proportion of red blood cells and then a second centrifugation step was performed with the second monovette for 10 min at 980g. After centrifugation the platelets accumulate at the bottom with the platelet-poor plasma (PPP) on top. The PPP was drawn off to separate hPRP from PPP. Before and after the preparation of hPRP, an aliquot was removed and the platelets in whole blood and hPRP were determined automatically by hematology analyzer (Sysmex KX-21, Sysmex, Japan). All procedures were preformed in super-clean bench. The platelets in each milliliter of hPRP sample were activated by 100 μL thrombin activators (1000 U bovine thrombin in 1000 μL 10% calcium chloride, prepared immediately before use) and the mixture was allowed to undergo maximal clot retraction at 4 °C overnight and then was centrifuged at 3000g for 10 min. The superstratum (hPRP-released growth factors) was collected, preserved with many aliquots, stored at −70 °C and thawed immediately before every change of the culture medium. All the in vitro studies were accomplished within 3 weeks to ensure the hPRP-released growth factors could maintain their biological effects.

2.4. Measurements of the levels of TGF-β1 and PDGF-AB

TGF-β1 levels were assayed using a commercially available ELISA kits (Pepro-Tech, NJ, USA). A dilution series of TGF-β1 standards was prepared in 100 μL volumes in 96-well microtiter plates coated with TGF-β-receptor II. The lower detection limit of TGF-β1 was 7 pg/mL. Since a large proportion of the TGF-β1 in biologic samples is often present in a latent form, conversion of TGF-β1 to its active form is necessary to estimate total TGF-β1. To do this, 0.5 mL of the hPRP samples were mixed with 0.1 mL of 1 M HCl, incubated at RT for 10 min, neutralized by addition of 0.1 mL of 1.2 M NaOH/0.5 M HEPES, and centrifuged. The supernatant fraction was then assayed for total TGF-β1 content. Aliquots (200 μL) were added in duplicate to the microtiter plate, which was then covered and incubated for 3 h at RT. The wells were then washed, enzyme-conjugated polyclonal antibody to TGF-β1 was added, and incubation continued for 1.5 h at RT. The plates were washed, substrate was added to each well, and the absorbance at 450 nm was determined for each using a microtiter plate reader. PDGF-AB levels were also tested following the manufacturer's protocol and mostly according to the measurement of TGF-β1. Triplicates were performed for all assays.

2.5. Proliferation and osteogenic differentiation of hADSCs stimulated by hPRP in vitro

The hADSCs were seeded in 96-well plates (Corning Life Sciences, Acton, MA, USA) at relatively low density (2×10^3 cells/well) for proliferation assay, and were seeded in 24-well plates (Corning Life Sciences, Acton, MA, USA) at relatively high density (2×10^4 cells/well) for analysis of alkaline phosphatase activity and mineralization. Eight groups including six groups of different concentrations of hPRP were set for the following three studies (see Table 1) and each group was in triplicate.

2.5.1. Study 1: assessment of the effect of hPRP on cell proliferation

The hADSCs were seeded in 96-well plates and divided into eight groups as above. Cell proliferation analysis was performed using the MTT assay. After culturing for 0, 2, 4, 6, 8, 10, and 12 days, the MTT assay was performed according to the cell proliferation kit protocol (Sigma). This assay is based on the ability of mitochondrial dehydrogenases to oxidize thiazolyl blue (MTT), a tetrazolium salt 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, to an insoluble blue formazan product. Then, the optical density (OD) of the plates was read on microplate reader (Bio-Rad Model 550, Hercules, CA, USA) using test and reference wavelengths of 540 and 620 nm, respectively. This test was repeated three times. The growth curves of hADSCs cultured in all groups were drawn and the absorbance of each group at day 4, exponential growth phase, was compared statistically.

2.5.2. Study 2: alkaline phosphatase activity of hPRP-induced hADSCs

The hADSCs were seeded in 24-well plates and divided into eight groups as above. The level of ALP activity was determined on days 7 and 14. Cells were rinsed two times with PBS followed by trypsinization and then scraped into ddH_2O. This was followed by three cycles of freezing and thawing. ALP activity was determined at 405 nm using p-nitrophenyl phosphate (pNPP) as the substrate. A 50-μL sample was mixed with 50 μL pNPP (1 mg/mL) in 1 M diethanolamine buffer containing 0.5 mM $MgCl_2$, pH 9.8 and incubated at 37 °C for 15 min on a bench shaker. The reaction was stopped by the addition of 25 μL of 3 M NaOH/100 μL of reaction mixture. Enzyme activity was quantified by absorbance measurements at 405 nm. Total protein content was determined with the BCA method in aliquots of the same samples with the PIERCE (Rockford, IL, USA) protein assay kit, read at 562 nm and calculated according to a series of albumin (BSA) standards. ALP levels were normalized to the total protein content at the end of the experiment. This test was repeated three times.

2.5.3. Study 3: mineralization assays for hPRP-induced hADSCs

The hADSCs were seeded in 24-well plates and divided into eight groups as above. Mineralization in osteoblast cultures was determined by staining of alizarin red S on days 14 and 21. For quantification of matrix calcification, plates were washed three times with PBS (pH 7.4), then stained with 0.5% alizarin red S in H_2O, pH 4.0, for 1 h at RT. After staining, cultures were washed three times with H_2O followed by 70% ethanol. To quantify matrix mineralization, alizarin red S-stained cultures were incubated in 100 mM cetylpyridinium chloride for 1 h to solubilize and release calcium-bound alizarin red S into solution. The absorbance of the released alizarin red S was measured at 562 nm. Data were expressed as units of alizarin red S

Table 1
Concentrations of hPRP and cells in different groups in studies 1, 2 and 3

Study	Groups	Description	Cell/well ($\times 10^3$)	Study	Groups	Description	Cell/well ($\times 10^4$)
1 (96-well plates)	1	Positive	2	2 and 3 (24-well plates)	1	Positive	2
	2	Negative	2		2	Negative	2
	3	2.5% hPRP	2		3	2.5% hPRP	2
	4	5.0% hPRP	2		4	5.0% hPRP	2
	5	7.5% hPRP	2		5	7.5% hPRP	2
	6	10.0% hPRP	2		6	10.0% hPRP	2
	7	12.5% hPRP	2		7	12.5% hPRP	2
	8	15.0% hPRP	2		8	15.0% hPRP	2

In study 1, positive group contained DMEM + 10% FBS + antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Negative group contained DMEM + antibiotics. In studies 2 and 3, positive group was traditional osteogenic media containing: DMEM + 10% FBS + 100 nM dexamethasone (DEX) + 0.2 mM ascorbic acid + 10 mM β-glycerophosphate + antibiotics. Negative group contained DMEM + 10% FBS + antibiotics. In studies 1, 2 and 3, all hPRP groups contained: DMEM + certain concentration of hPRP + antibiotics.

released (1 unit = 1 unit of optical density at 562 nm) per milligram of protein in each culture on a parallel well. This test was repeated three times.

2.6. Construction of ITB

hADSCs were induced by osteogenic medium, containing 12.5% hPRP + 100 nM dexamethasone (DEX) + 0.2 mM ascorbic acid + 10 mM β-glycerophosphate, for 1 week before SEM and in vivo study. hPRP (0.4 mL) induced hADSCs (6×10^5 cells) were aspirated into a 1-mL syringe. Here the cells were resuspended directly into PRP. In a second 1-mL syringe, 100 μL thrombin activators were aspirated. 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 expel from the two syringes. The third channel of the "T" connector was connected with a puncture needle. When applying, the two plungers were gently pushed together, allowing the two components to mix adequately.

2.7. Examination of ITB construct through SEM

Samples were fixed overnight in cacodylate buffered 2.5% glutaraldehyde at 4 °C, then the specimens were postfixed in 1% OsO_4 for 1.5 h, dehydrated with a series of ethanols, dried in a critical point dryer (VG Microtech, East Grinstead, UK), mounted onto aluminum stubs, sputter coated with gold, and viewed under a scanning electron microscope (JEOL 840, Peabody, MA).

2.8. Investigation of the osteogenic potential of ITB in vivo

For in vivo evaluation, 4–6-week-old, 16 BALB/c homozygous nude (nu/nu) mice were used (Peking University Experimental Animal Center). All animal experiments were performed in accordance with the institutional animal guidelines. Animals were divided into two groups. In group 1, one side of the inguinal groove of nude mice was subcutaneously injected with ITB (experimental side). The other side was injected with induced hADSC (6×10^5 cells) resuspended in 0.4 mL PBS as control (control side). In group 2, ITB was injected as group 1 and the control was 0.4 mL hPRP only. Specimens of each group were harvested at 4 weeks after injection and animals in each group were sacrificed by CO_2 asphyxiation. X-ray examinations were used to evaluate the mineral density of ITB and the constructions were carefully removed and fixed in 4% paraformaldehyde. Then, the samples were decalcified for 10 days in 10% EDTA (pH 7.4). After decalcification, the specimens were dehydrated and subsequently embedded in paraffin. Sections (5 μm thickness) were stained with hematoxylin and eosin (H&E). Osteogenesis was evaluated with immunohistochemical analysis for Collagen I, Osteopontin (OPN) and Osteocalcin (OC) (sp kit, VECTOR, USA, primary antibody were purchased from Santa Cruz). Specimens were processed using identical protocols, while the specimens without adding the primary antibodies were used as the negative control and human osteoblasts were used as the positive control.

2.9. Statistical analysis

Data were analysed using SPSS version 10.0 (Chicago, IL, USA). Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) and post hoc test for multiple comparisons was carried out using the Fisher LSD test. When variance was not homogeneous, the Kruskal–Wallis test was used, followed by the Nemenyi test for multiple comparisons. For all tests, statistical significances were accepted for P values lower than 0.01.

3. Results

3.1. Platelet counts and levels of growth factors

The platelet counts in hPRP preparations were 10.3×10^8/mL for sample A and 11.0×10^8/mL for sample B. More details were showed in Table 2. Sample A was applied during experiments in vitro and sample B was used for repeated experiments and in vivo studies.

3.2. Study 1: the effects of different concentrations of hPRP on cell proliferation

The effects of different concentrations of hPRP on hADSCs' proliferation were shown as growth curves (Fig. 1) and the OD of each group at 96 h was statistically compared (Fig. 2). There was a statistically significant increase in hADSCs' proliferation when the cultural media contained 10% FBS (positive group) or 2.5–15% hPRP compared with the negative control ($P < 0.01$). The promoting effects of 5–15% hPRP on cell proliferation were significantly higher than that of the positive control ($P < 0.01$) and that of the 12.5% hPRP group was significantly higher than that of other hPRP concentration groups ($P < 0.01$).

3.3. Study 2: alkaline phosphatase activity of hADSCs cultured by different concentrations of hPRP

The ALP activity of hADSCs in response to different hPRP concentrations was increased in a dose-dependent mode of action, as indicated in Fig. 3a and b. Following 7 days and 14 days of exposure to the various concentrations of hPRP, there were statistically significant increases in ALP activities of the groups containing 10 and 12.5% hPRP compared with the negative control ($P < 0.01$). When cultured for 7 days, compared with the positive control, there were no statistical differences of ALP activities for the groups containing 10 and 12.5% hPRP ($P > 0.05$) and the ALP activities of other hPRP groups were significantly lower than that of the positive control ($P < 0.01$). When cultured for 14 days, compared with the positive control, there were no statistical differences of ALP activities for the groups containing 7.5, 10, 12.5% and 15% hPRP ($P > 0.05$) and the ALP activities of other hPRP groups were significantly lower than that of the positive control ($P < 0.01$).

3.4. Study 3: the mineralization of hADSCs induced by different concentrations of hPRP

The effects of different hPRP concentrations on the mineralization of induced hADSCs were shown in Fig. 4a and b. When cultured for 14 days, there were statistically significant increases in the mineralization of induced hADSCs when the cultural media contained 7.5–15% hPRP compared with the negative control ($P < 0.01$) and that of the 12.5% hPRP group was significantly higher than other hPRP concentration groups ($P < 0.01$). When cultured for 21 days, there were statistically significant increases in the mineralization of induced hADSCs when the cultural media contained 5–15% hPRP compared with the negative control ($P < 0.01$) and that of the 10 and 12.5% hPRP groups were significantly higher than other hPRP concentration groups ($P < 0.01$). However, following 14 days or 21 days culturing, the effects of all hPRP groups on the mineralization were significantly lower than that of positive control ($P < 0.01$).

3.5. Morphology of ITB under SEM

Well-proportioned hADSCs were enwrapped in a three-dimensional scaffold of fibrin which came from the fibrinogen of hPRP. The activated and non-activated platelets embedded in the interspaces of the scaffold around the cells (Fig. 5a and b).

3.6. Results of in vivo study

After 4 weeks' breeding, subcutaneous masses in relatively hard texture were detected in the experimental sides of the nude mice in the two groups. In X-ray radiography, the mass (osteoid formation) was observed in relatively high but inhomogeneous density. The gross observation showed that the osteoid formation, without

Table 2
Platelet counts and the levels of growth factors in whole blood and hPRP samples

Samples	Whole blood (10^8/ml)	hPRP (10^8/ml)	hPRP/whole blood ratio	TGF-β1 (ng/ml)	PDGF-AB (ng/ml)
A	1.21	10.3	8.5	393.71	112.68
B	1.39	11.0	7.9	375.96	79.82
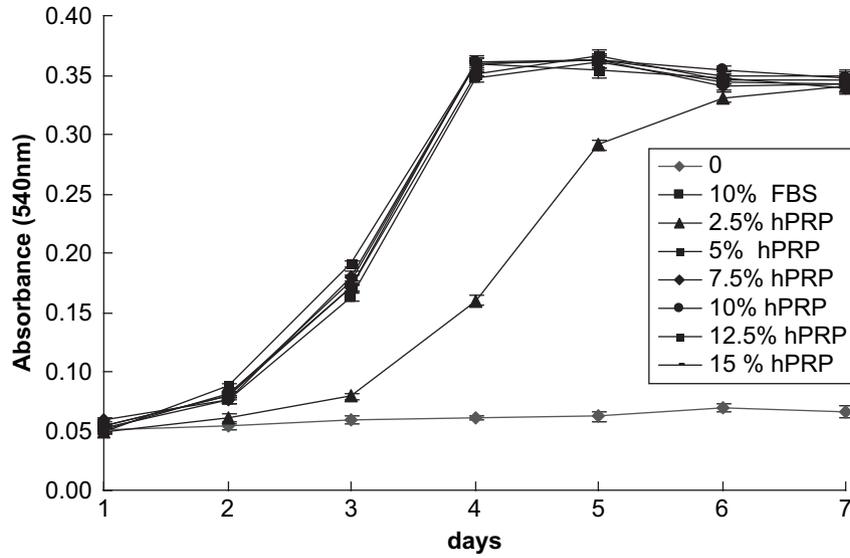
Fig. 1. The growth curves of hADSCs cultured by different concentrations of hPRP.

fibrous tissue capsule, adhered to adjacent muscles and subcutaneous tissue in experimental sides (Fig. 6a–d). Histological examination of the specimens using hematoxylin and eosin stains revealed that neogenetic bone-like structure integrated mutually to form matured woven bone and bone trabecula (Fig. 7). Immunohistochemistry showed that Collagen I (Con I), Osteopontin (OPN) and Osteocalcin (OC) expressed in the bone-like structure areas, displaying dark yellow granules (Fig. 8a–c). In contrast, no new tissue mass or structure was found in the control sides of the two groups under X-ray detection and gross observation.

4. Discussion

To realize the idea of "biological solutions to biological and medical problems", a novel ITB composed of hPRP and hADSCs was constructed in this study. Obviously, this ITB is more feasible for clinical use because the two ingredients could be easily obtained from autologous resources with large quantity and minimal donor site morbidity. Moreover, in the cell proliferation and osteogenic inducing procedures, hPRP was used to eliminate the influence of foreign protein (fetal bovine serum) and the liquid hPRP and hADSCs could be applied by injection and grafted in minimally invasive way. However, there was still no systematic study on the effects of hPRP on the proliferation and differentiation of hADSCs in vitro and in vivo. Therefore, the purpose of this study was to explore the optimal concentration of hPRP in the culture media of hADSCs in vitro and the osteogenic potential of the ITB in vivo.

4.1. The effects of different concentrations of hPRP on the proliferation and osteogenic differentiation of hADSCs

Nowadays, many researches have been focused on the use of the pool of growth factors released from hPRP as a powerful substitute of fetal bovine serum (FBS) in the culture of various kinds of cells,
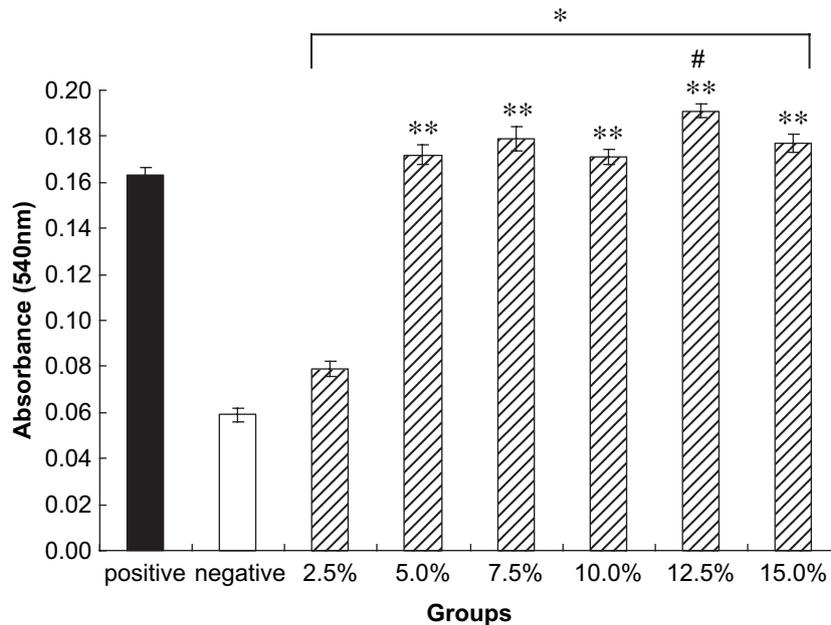
Fig. 2. OD of each concentration group at 96 h. *: $P < 0.01$ vs negative group. **: $P < 0.01$ vs positive group. #: $P < 0.01$ vs other concentration groups (the Fisher LSD test).
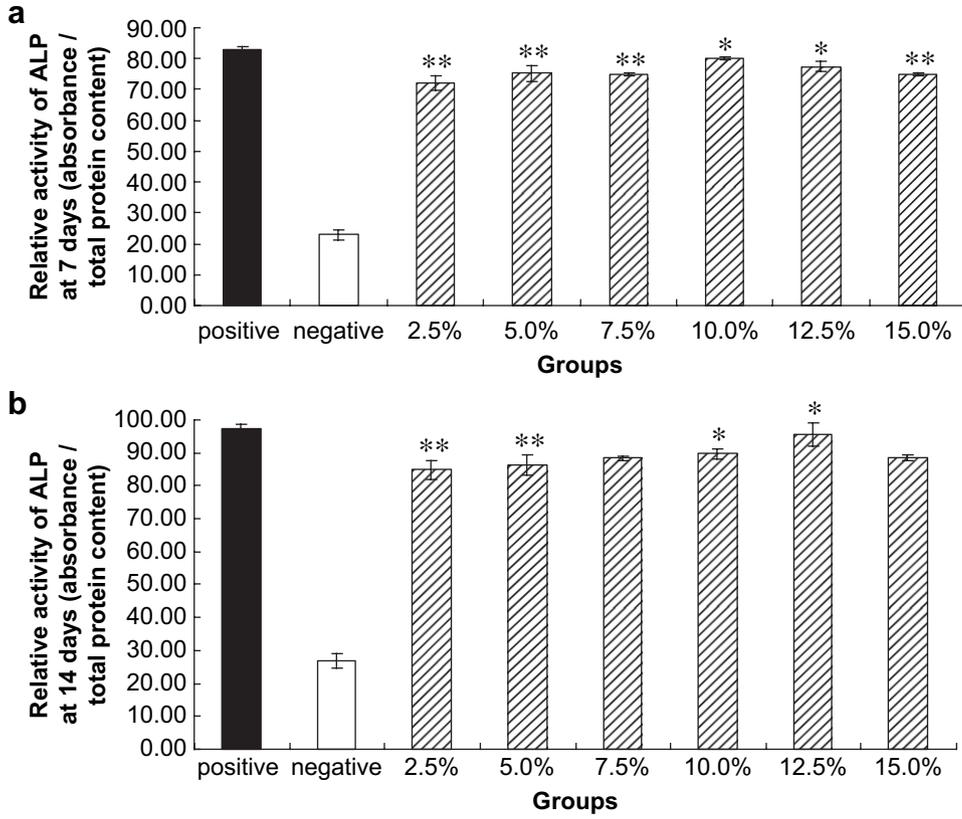
Fig. 3. (a) Relative activities of ALP of different concentration groups at day 7. (b) Relative activities of ALP of different concentration groups at day 14. *: $P < 0.01$ vs negative group. **: $P < 0.01$ vs positive group (the Nemenyi test).
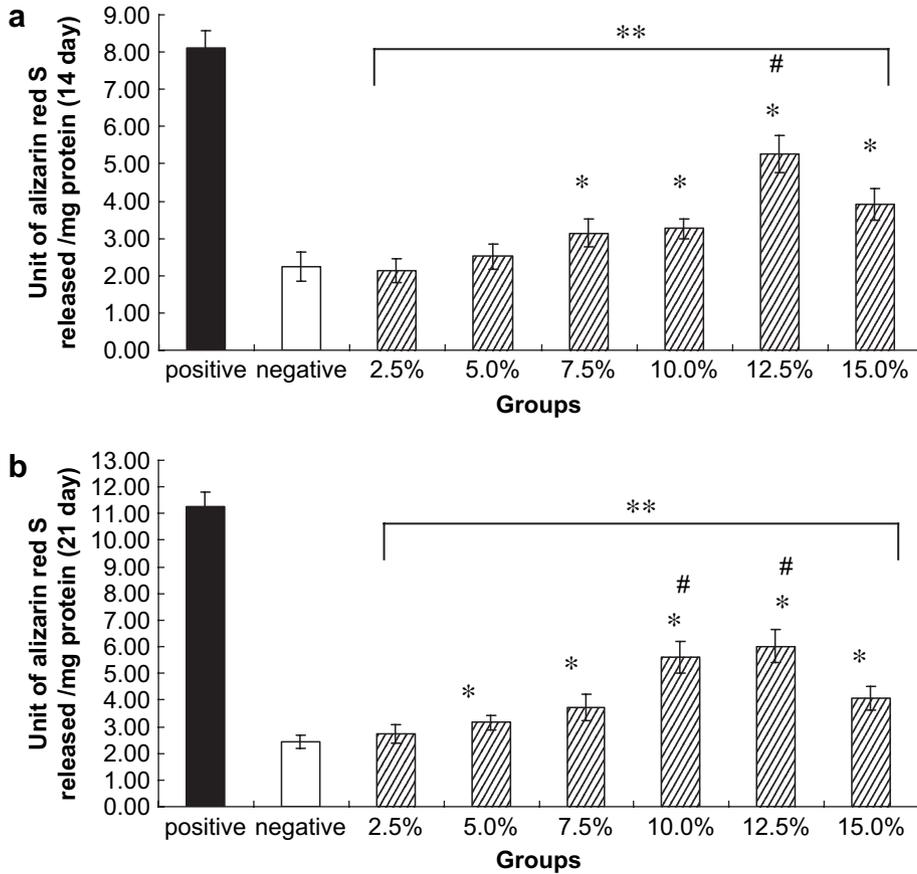
Fig. 4. (a) Mineralization in cultures of different concentration groups at day 14. (b) Mineralization in cultures of different concentration groups at day 21. *: $P < 0.01$ vs negative group. **: $P < 0.01$ vs positive group. #: $P < 0.01$ vs other concentration groups (the Fisher LSD test).
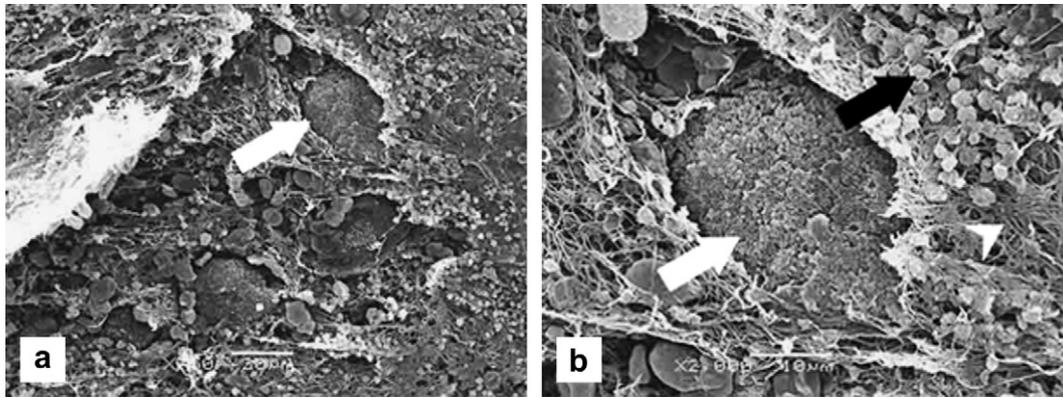
Fig. 5. ITB constructs as observed by SEM. (a) hADSCs (white arrow) were enwrapped by fibrin (×750). (b) hADSCs (white arrow) and the activated platelets (black arrow) were embedded in the interspaces of the fibrin scaffold (white arrow head) (×2000).

including mesenchymal stem cells [14], stromal cells [15], fibroblasts and osteoblasts [16]. However, there were still conflictive outcomes among different studies [14–16]. The reasons regarding these contradictions might exist in the percentage of PRP in the cultural medium [13,17]. In this study, cultural media containing 2.5–15% hPRP were used to incubate hADSCs and the proliferation and differentiation of the cells in different groups were compared statistically. The concentrations of TGF-β1 and PDGF-AB of the hPRP used in this study were tested quantitatively using ELISA assay. Therefore, the concentration of the representational growth factors in each group could be calculated. The quantitative study avoided the influence of the differences in hPRP's preparation, facilitated the repeated experiments and made it possible for other researchers to make reference to this study. Obviously, the biological effects of hPRP were dose-dependent and 10 and 12.5% seemed to be ideal concentrations. The results about the dose-dependent property of hPRP were similar to the previous studies [13,17,18]. In the following *in vivo* study, to make the most of the effects of hPRP

on hADSCs, the cells were cultured in the media containing 12.5% hPRP since the primary passage. Through the experiments, the effect of hPRP on the proliferation of hADSCs was prominent, even the effects could be higher than the positive control (DMEM + 10% FBS). However, the effect on the osteogenic differentiation of hADSCs showed relatively limited especially in the mineralization assay. Therefore, before the following *in vivo* study, osteogenic differentiation ability of hADSCs was strengthened by culturing in osteogenic medium (12.5% hPRP + 100 nM DEX + 0.2 mM ascorbic acid + 10 mM β-glycerophosphate) for 1 week.

hPRP could simulate hADSCs proliferation and osteogenic differentiation and the mechanism of the biological effects might consist in: PDGF could activate the mitogenesis of endothelium, fibroblasts, macrophages, and marrow stem cells [7] and stimulate cells to synthesis type I collagen [19]. The combination of PDGF with IGF-I has been approved effective in promoting bone regeneration [20]. TGF-β is general growth and differentiating factor involved with bone regeneration in mitogenesis of osteoblast
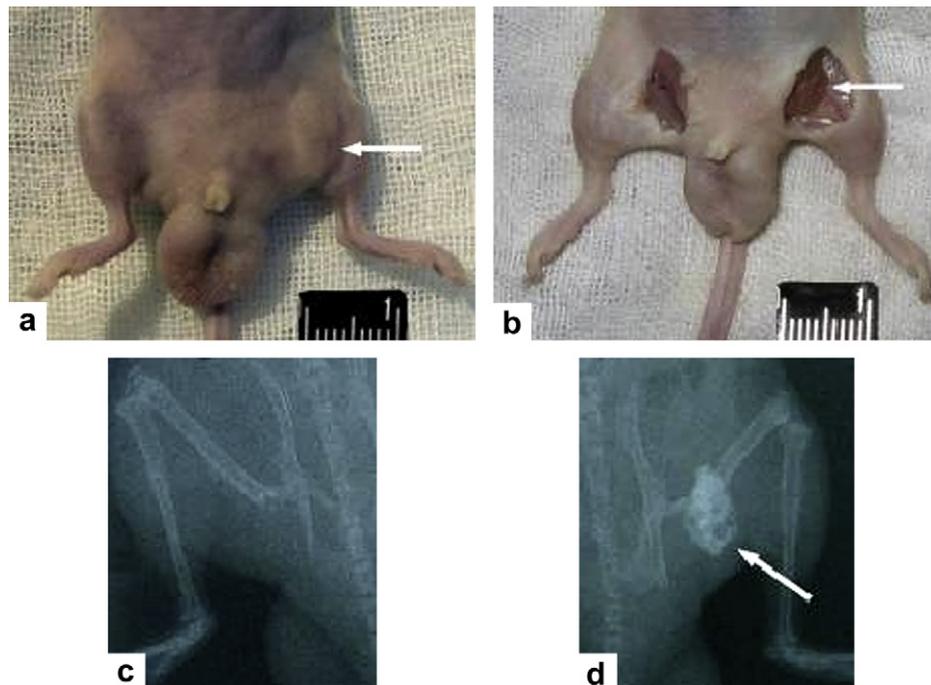
Fig. 6. A subcutaneous mass of X-ray obstacle was found in experimental side of nude mice and no positive result was found in control side. (a) Gross observation of the subcutaneous mass (white arrow). (b) Gross observation of the subcutaneous mass after dissection (white arrow). (c) No positive result was found in control side by X-ray observation. (d) X-ray obstacle was found in experimental side.
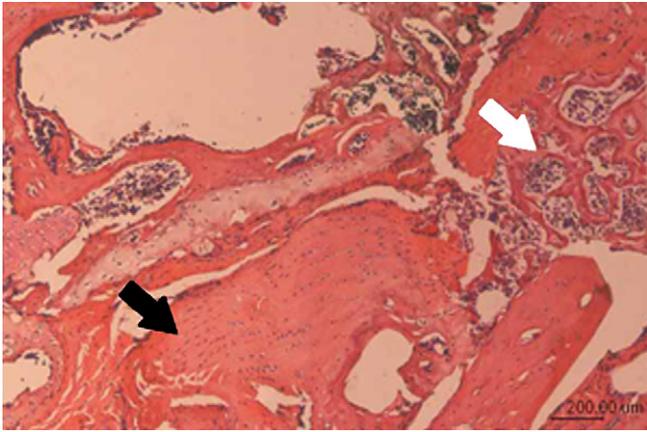
Fig. 7. H&E staining indicated bone structure formation. Typical bone structure (black arrow) and bone trabecula-like structure (white arrow) were observed (×40).

precursors [21]. In high concentration, it could enhance DNA synthesis of bone cells and induce matrix synthesis of mesenchymal cells and angiogenesis [22]. Additionally, it could inhibit osteoclast formation and bone resorption [14]. In the *in vivo* study, when injected subcutaneously, PRP in ITB was activated and formed a coagulated mass. In succession, the degranulation of the alpha granules of platelet occurred and a circumstance rich in growth factors was created. The high concentration of PRP-released growth factors, which were short-term but essential for the progression of coordinated wound healing and improved vascularity and tissue regeneration, was supposed to have a positive effect in the early phase of bone regeneration [23–25].

4.2. The feasibility and advantages of the injectable tissue-engineered bone

Among the existing literatures, the researchers made efforts to accelerate bone restoration by using PRP combined only with autologous bone [26], organic bone-graft substitute [27], or inorganic biomaterial [28]. However, no, or not enough vital cells (osteoblasts or circulating stem cells) in these substitutes or scaffold might result in low efficiency for large bone defects [29]. As an ideal tissue-engineered bone construct, not only osteogenic growth factors and scaffold but also seed cells should be contained.

In our *in vivo* study, hADSCs were used as seed cells, hPRP-released growth factors were used as osteogenic growth factors and the hPRP fibrin reticulation was used as autologous scaffold. hADSCs, as mentioned above, are available in large quantities of stem cells at harvest, can decrease the time consumption, and reduce the risk of contamination. hPRP, when mixed with thrombin activator, can release growth factors and become gel-like [30]. The hPRP-released growth factors are conducive for inducing osteogenic differentiation and the gel-like fibrin scaffold can sustain a certain volume and be applied as carrier for growth factors and cells [31,32]. Also this scaffold is easier to be absorbed and exhibits excellent plasticity [5]. SEM examination showed that hADSCs and platelets were enwrapped by fibrin. The fibrin could maintain the regenerative space and provide a matrix for cell migration and proliferation [29].

When the ITB in this study was injected by bi-connected injector subcutaneously, hPRP-released growth factors as osteogenic growth factors might take on importance for the initial 7–10 days. The combination of PDGF, TGF-β, and IGF could stimulate the chemotaxis and mitogenesis of adjacent stem cells and osteoblasts, angiogenesis for capillary ingrowth, bone matrix formation, and collagen synthesis [7,30]. In the following phase, the cytokines and growth factors were presumed to be secreted by induced hADSCs and other cellular sources nearby. These events would reinforce the concentration of necessary growth factors in the microenvironment and improve bone regeneration locally [33]. Therefore, the seed cells in tissue-engineered material might play a vital role in the process of bone regeneration [34,35].

The liquid ITB in this study was convenient to operate and especially appropriate for irregular defects. hADSCs and hPRP, the elements of ITB, could be easily acquired from autologous resources and avoid transplantation rejection, diseases transmission and
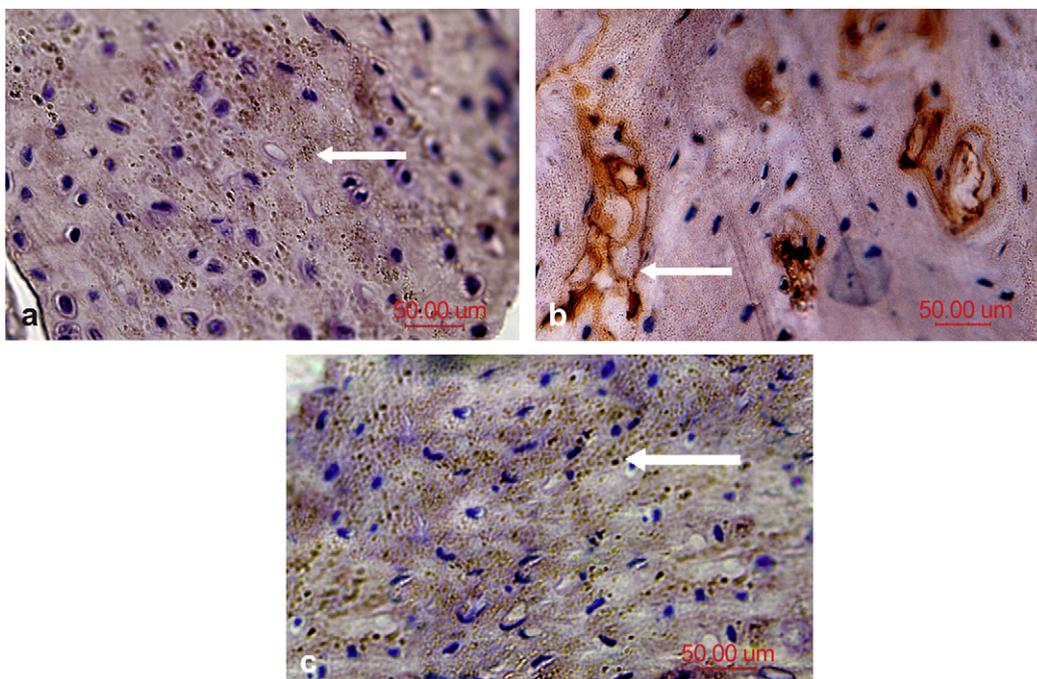
Fig. 8. Immunohistochemistry staining. Dark brown granules which showed the positive results could be seen (white arrow, ×200). (a) Con I. (b) OPN. (c) OC.

ethical issues. Also, ITB could be grafted in minimally invasive way and easily accepted by patients.

5. Conclusions

The *in vitro* experiments indicated that human platelet-rich plasma could induce the proliferation and osteogenic differentiation of human adipose-derived stromal cells and 10–12.5% of human platelet-rich plasma seemed to be the optimal concentration. The *in vivo* study showed that the novel injectable tissue-engineered bone could form bone structure in heterotopic site of nude mice and could represent a prologue for the development of a new biological solution to bone defect. However, further *in vivo* investigations should be done in the future to fully reveal the characteristics of this injectable tissue-engineered bone.

Acknowledgements

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