Effects of sustained release Platelet-rich plasma on bone healing in critical-size defect

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The aim of this study was to investigate and compare the osteogenetic effects of different types of platelet-rich plasma (PRP)-loaded alginate scaffolds on radius defects in rabbits. A critical size defect (15mm in length) was created in the mid-upper part of the bilateral radius of 46 New Zealand White rabbits. The rabbits were supplied with PRP alginate beads (14 rabbits), PRP alginate capsules (14 rabbits), PRP gel (14 rabbits), or the defect was left untreated (4 rabbits). At 8 and 12 weeks after implantation, radiographic and histological observations were performed to investigate the bone healing of the defect. The results showed that each material's ability to produce new bone formation was arranged in the following order: PRP alginate beads＞PRP alginate capsules＞PRP gel, with significant differences between each material ($P<0.01$). PRP alginate beads could be a promising material capable of enhancing bone regeneration.

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PRP is a volume of autologous plasma with a platelet (thrombocyte) concentration above baseline (1×10^9 pl/ml) [1]. The growth factors released from platelets have been shown to include platelet-derived growth factor (PDGF) [2], transforming growth factor-(TGF-) beta, platelet-derived epidermal growth factor (PDEGF), platelet-derived angiogenesis factor (PDAF)[3], insulin-like growth factor (IGF-1), platelet factor-4 (PF-4) [2,4,5], and vascular endothelial growth factor (VEGF) [6,7]. Many of these growth factos play critical roles in stimulating cell proliferation [8], the osteogenic differentiation of mesenchymal stem cells [9] and angiogenesis, which is necessary for bone formation and remodeling [10]. Hence, PRP applications have been gaining considerable popularity in the fields of orthopaedic, maxillofacial, and periodontical surgery [11-16] due to PRP's potential efficacy in augmenting bone formation.

The clinical evidence for PRP as a therapeutic agent is insufficient due to obstacles such as rapid washout and inactivation of the critical proteins in PRP [17]. A large body of data through in vitro and in vivo studies[17-26] using PRP for bone healing has failed to demonstrate efficacy unless PRP is used in conjunction with an osteoconductive biomaterial that controls the bioavailability of GFs in PRP. Various biomaterials, such as heparin-conjugated fibrin (HCF), gelatine, and chitosan, have been utilised as PRP sustained delivery carriers to prevent initial washout and control the release of important proteins in PRP to the target area. Alginate is a well-characterised biopolymer that has the advantages of being biocompatible [25,27-29], osteoconductive [30-33], nonimmunogenic [25,29], nontoxic [34], and biodegradable [25,29]. Previously, Sylvia et al [25] designed an alginate hydrogel-based PRP delivery system that included two types of PRP-alginate carriers: PRP-alginate beads and PRP-alginate capsules. In vitro studies performed by Sylvia et al [25] and Helen et al [26] compared and evaluated the effects of the two carrier types on the growth and osteogenic differentiation of both human mesenchymal stem cells and human osteoblast-like cells. The studies found that the cellular response was carrier type dependent. However, to date, no conclusion has been reached regarding which carrier type performs best. To our knowledge, there are currently no reported studies aimed at comparing the effects of both PRP-alginate beads and PRP-alginate capsules on tissue-engineered bone formation through in vivo animal studies.

We hypothesised that the biological characteristics of PRP alginate composites are superior to those of PRP gel in bone engineering and that different preparation techniques for PRP alginate composites result in siginificant differences in bone repair. Our overall study objective can be outlined as follows: (1) to investigate the potential of alginate hydrogel as a biomaterial for

controlled release of multiple growth factors present in PRP and to determine whether the combination of PRP and alginate results in better bone formation than PRP gel alone; (2) to compare the bone-regenerating effects of two different types of PRP-loaded alginate scaffolds for tissue engineering applications and to determine which material can be further developed for clinical use.

1. Materials and methods

Animal models

Forty-six 6-month-old New Zealand White rabbits (approximate weight, 2.5-3.5 kg) were utilised as experimental subjects. The animals were kept in separate cages, fed a standard diet, and allowed to move freely during the study. All rabbits remained in good condition throughout the duration of the study. The ethics committee of the First Affiliated Hospital of Harbin Medical University approved all experimental procedures.

Preparation of PRP

PRP was prepared according to established procedures [7]. Specifically, 10 mL of venous blood was freshly obtained from 42 rabbits using a syringe containing 1 mL of acid citrate dextrose (ACD) solution as an anticoagulant. The mixture was centrifuged in a laboratory centrifugation apparatus (ACE Surgical Supply Company, USA) at 4°C for 15 min at 200×g. Subsequently, the yellow plasma containing the platelet fraction and the buffy coat layers were collected and further spun at 4°C for 10 min at 200×g to separate the platelets. After discarding the platelet-poor plasma, the lower half of the plasma and the pellet were resuspended and collected as the PRP. Approximately 1 mL of PRP was obtained. Platelet counts were performed for the PRP samples using an automated haematology analyser (ABX company, France). This preparation method yielded an average platelet count of 0.5 to 1.0×10^6 platelets/μL.

Preparation of three composite types

The composite was prepared as follows. PRP was conventionally gelatinised to a platelet gel conventionally by mixing 1 mL PRP solution and 0.167 mL mixture of 10% calcium chloride and 300 E bovine thrombin (Fibriquick, Netherlands) under sterile conditions, according to a previously reported procedure [1]. PRP alginate beads and capsules were produced according to a previously reported method [26]. Alginate beads containing PRP were formed via the internal gelation process. The PRP +alginate mixture was then dispensed via a syringe needle (261/2 gauge) into 6% $CaCl_2$(Sigma, America). After gelation, the beads were incubated in $CaCl_2$ solution for 5 min to complete the gelatine process. Conversely, the alginate capsules with PRP were formed via the external gelation process. PRP was first combined with a 6% $CaCl_2$ solution (2:5 volume ratio; Sigma), and the mixture was then dispensed dropwise through a syringe needle (261/2 gauge) into a stirring 1% alginate solution. After gelation, the capsules were stirred in the $CaCl_2$ solution for 5 min to ensure the completion of the gelation process.

Surgical procedure

The in vivo experiment was performed using a previously reported surgical procedure [1]. The operation site, either the left or right front limb, was shaved, prepared, and draped for aseptic surgery while the rabbits were in the supine position. A 4.5-cm-long superomedial incision was made, and the tissue overlying the diaphysis of the radius was dissected. A 1.5-cm segmental defect was prepared in the radius using a surgical oscillating saw supplemented by copious sterile saline water irrigation. PRP alginate beads, PRP alginate capsules, and PRP gel were applied to some of the defects, while other defects were left untreated. Fixation of the ostectomised bone was unnecessary because of the fibro-osseous union between the ulna and radius located distal and proximal to the surgical site. The soft tissue was approximated with interrupted 4-0 sutures (Ethicon, USA), and the skin was closed with 3-0 silk sutures (Ethicon, USA). A postoperative antibiotic (fostomycin) (Meiji Seika, Japan) was administered intramuscularly at a dose of 100 mg/kg per day for 3 days.

Scanning electron microscopy observation

Surface and cross-section morphologies of PRP gel, PRP-alginate beads and capsules were examined using a scanning electron microscope (SEM; S-3400N, Hitachi, Japan). The scaffolds were gently washed with PBS and fixed with 2.5% (vol/vol) glutaraldehyde (Sigma) in 0.1 M PBS (pH 7.4) for 24 h at 4°C. After being thoroughly washed with PBS, the scaffolds were dehydrated in a graded ethanol series (30, 50, 70, 90 and 100%, vol/vol). After the specimens were dehydrated twice in each ethanol concentration (15 min each time), the scaffolds were freeze-dried, coated with a gold-palladium layer, and then visualised by SEM.

Radiologic assessment

All animals were euthanised at 8 or 12 postoperative weeks. The radius-ulna complex containing the defect was then removed and fixed in 10w% formaldehyde solution in PBS for 24 h for the assessment of bone regeneration. Bone regeneration at the bone defect site was assessed by X-ray (MX-20 Film;Faxitron company, America) and Brilliance iCT (Philip, Netherlands) at the 8th and 12th weeks post-injury. The sums of the bone formation, proximal union, distal union, and remodelling scores were analysed and compared between groups. The results were scored using the modified Lane and Sandhu scoring system (Table 1) [35].

Histological analysis

Bone specimens were subsequently decalcified in 10% ethylenediaminotetraacetic acid (EDTA) solution after radiographic analysis. Following decalcification for approximately 6 weeks, the specimens were processed for paraffin embedding. Serial sections (4-μm-thick) were cut

in a longitudinal direction. Half of the sections were stained with hematoxylin and eosin, and the others were stained with Masson's trichrome.

Statistical analysis

The data normality and homogeneity of variances were verified. All values were expressed as the mean±standard deviation. Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., USA). The radiographic scores were evaluated. Significant differences between groups were determined by an analysis of variance (ANOVA), followed by a post hoc Turkey's test when the ANOVA suggested a significant difference between groups ($P<0.05$).

2. Results

SEM images analyses

As evident from the SEM images in Fig. 1, platelets and extending pseudopodia were successfully incorporated into the PRP-alginate composite scaffolds' porous structure. The three types of gelatinous biomaterials were visible in the typical three-dimensional porous network structure. A large amount of fibre inside the biomaterials interweaved and formed the scaffolds, which contained an abundant supply of interconnected space. The pore sizes of the PRP-alginate bead scaffolds [Fig. 1(a, a')] were larger compared with those of the PRP-alginate capsules scaffolds [Fig. 1(b, b')]. The pore structure in the PRP-alginate beads group was dense; in contrast, the pore structure in the PRP-alginate capsules group was loose. The surface morphology of the PRP-alginate bead scaffolds was regular and clear, whereas the surface morphology of the PRP-alginate capsule scaffolds was irregular and disorganised. The PRP-alginate bead scaffolds were superior compared with those of the PRP-alginate capsules and the PRP gel for surface morphology and adsorption of the platelets.

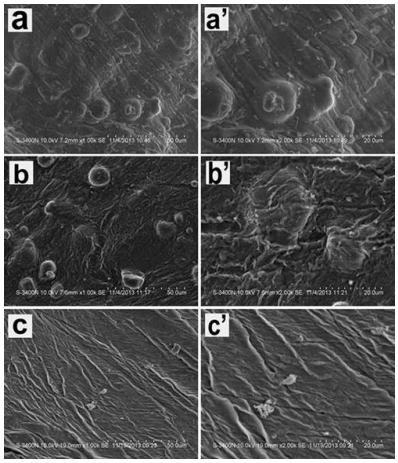

Fig. 1. SEM photographs of PRP scaffolds: (a, a') PRP-alginate beads (×1000, ×2000); (b, b') PRP-alginate capsules (×1000, ×2000) and (c, c') PRP gel (×1000, ×2000).

Radiographic analyses

Figs. 2 and 3 shows X-ray and iCT photographs of bone defects at 8 and 12 weeks after the implantation of the three biomaterial types. When the bone defect was treated with PRP-alginate composites and PRP gel, bone regeneration at the defect was radiographically detected; however, the extent of the radiopaque area was greater for the former than for the latter. Conversely, no radiographic bone formation was observed at the bone defect in the control group without any treatment. Healing of the bone defect in the PRP alginate bead group was superior to that of the PRP alginate capsule group and the PRP gel group. This finding demonstrated that there were radiologically significant differences in bone defect healing, formed in response to the different gels, among the animals in all three study groups at 8 and 12 weeks post-injury.

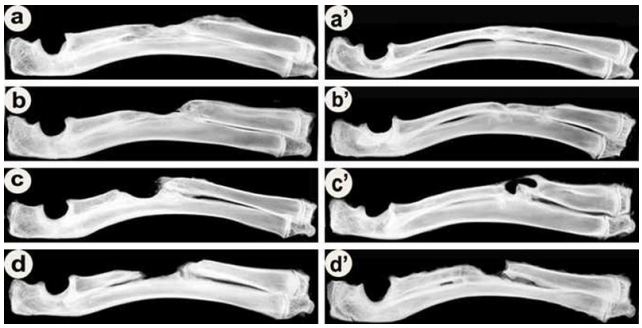

Fig. 2. X-ray photographs after implantation of different treatments into radial bone defects at various postoperative time points. PRP-alginate beads group at 8 weeks (a) and at 12 weeks (a'); PRP-alginate capsules group at 8 weeks (b) and at 12 weeks (b'); PRP gel group at 8 weeks (c) and at 12 weeks (c'); Blank control group (defect without any treatment) at 8 weeks (d) and at 12 weeks (d').

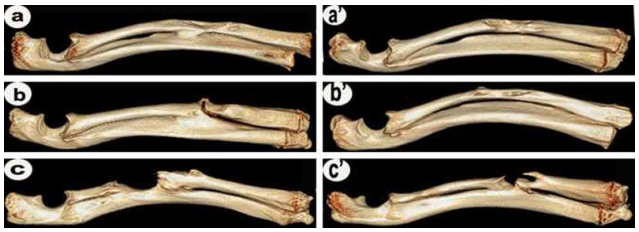

Fig. 3. CT photographs after implantation of different groups into radial bone defects.PRP-alginate beads group at 8 weeks (a) and at 12 weeks (a'); PRP-alginate capsules group at 8 weeks (b) and at 12 weeks (b'); PRP gel group at 8 weeks (c) and at 12 weeks (c').

Fig. 4 shows the radial defects' radiographic scores at 8 and 12 weeks after treatment with three different types of biomaterials. At weeks 8 and 12, the radiographic scores in the PRP-alginate beads group were significantly

higher than were those in any other group. An extensive analysis of variance revealed that the radiographic scores were significantly different among each of the three groups at each time point (n=7, P＜0.05).

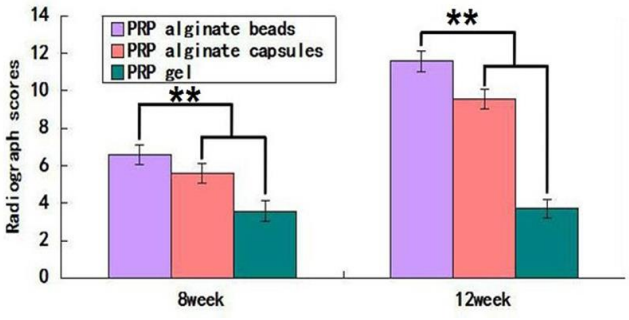

*Fig. 4. Radiograph scores of repaired rabbit radial defects in three groups (PRP-alginate beads group, PRP-alginate capsules group and PRP gel group) at 8 weeks and 12 weeks. **P<0.01 for the comparison among the three study groups.*

Histological analyses

Figs. 5 and 6 show histological sections of radius defects at 8 and 12 weeks after the application of PRP-alginate beads (Figs. 5 and 6a, a'), PRP-alginate capsules (Figs. 5 and 6 b, b'), or PRP gel (Figs. 5 and 6c, c'). When PRP-alginate beads were applied, the bone defect was histologically closed by newly regenerated bone tissue. Bone regeneration at the defect treated with PRP-alginate capsules was also observed, although the area of newly regenerated bone tissue was smaller. In contrast, less bone regeneration was observed with PRP gel treatment, while a remarkable ingrowth of cartilage and soft connective tissue into the defect was observed. These results were similar to those of the radiographic analysis.

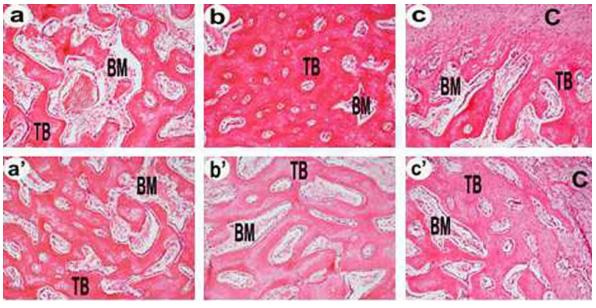

Fig. 5. Histological evaluation of all grafted sites after application of different treatments into radius bone defects at 8 weeks and 12 weeks (H&E staining, magnification ×100): PRP-alginate beads group at 8 weeks (a) and at 12 weeks (a'); PRP-alginate capsules group at 8 weeks (b) and at 12 weeks (b'); PRP gel group at 8 weeks (c) and at 12 weeks (c'). BT indicates bone trabecula; Ma, bone marrow; C, cartilage.

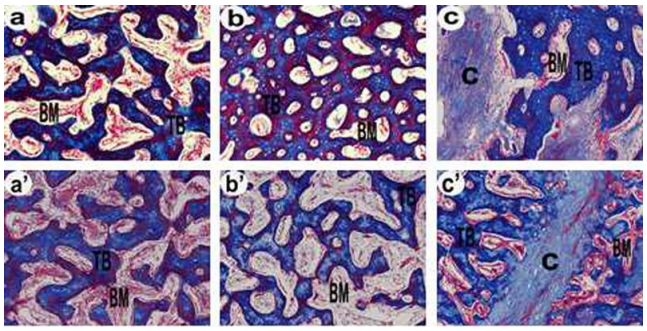

Fig. 6. Histological evaluation of all grafted sites after implantation of different treatments into radius bone defects at 8 weeks and 12 weeks (Masson staining, magnification ×100): PRP-alginate beads group at 8 weeks (a) and at 12 weeks (a'); PRP-alginate capsules group at 8 weeks (b) and at 12 weeks (b'); PRP gel group at 8 weeks (c) and at 12 weeks (c'). BT indicates bone trabecula; BM, bone marrow; C, cartilage.

3. Discussion

The present study used 15-mm-long radius defects to evaluate bone regeneration in adult rabbits (approximately 20 weeks old). It is well known that the establishment of specific bone defects in animal models is fundamental and crucial for bone tissue engineering research. Animal models of bone defects include rats, dogs, rabbits, sheep and monkeys; as a mammal, the bone defect rate of rabbits is similar to that of humans. Widely used sites for bone defect models include the skull, mandible, and limbs; the most commonly employed among these are the limbs, in which the radius and ulna are the most generally used. The rabbit radius is classified as a non-weight-bearing bone, while the ulna is defined as a weight-bearing bone. Because the radius and ulna exhibit mutual support, the stability of bone healing sites can be maintained after independent osteotomy of the radius, even without any external fixation methods. The radius thus could be a more appropriate site for bone defect models for bone defect models compared with the ulna. Critical size defects (CSD) are defined as defects of a size that will not heal spontaneously during the lifetime of the animal [36]. There has been much debate in the literature regarding the appropriate size of defects for bone regeneration in radius models. The size defect employed in the present study was in accordance with the critical size defect of 15mm recommended for rabbits[37], and bone healing was not observed without any treatment (Fig. 2 d, d'). The full-thickness, 15-mm-long radius defects that we aplied in the present study thus fulfil the criteria for a critical size bone defect, and the 15-mm-long radius defect model in adult rabbits is an appropriate model to evaluate bone regeneration.

The regenerative effectiveness of PRP products may be undermined due to rapid release, a short half-life, the

dilution of GFs within PRP, and the fact that the products are biodegradable. Because PRP-derived GF delivery is inefficient, PRP must be applied in combination with sustained release delivery carriers to reinforce the bioavailability of the GFs and the regeneration efficiency of the PRP products in clinical use. Recently, the selection and modification of PRP sustained release carriers has attracted great interest in regenerative medicine. The materials used to prepare sustained release GFs carriers can be divided into two categories: natural polymers and synthetic polymers. Natural polymeric materials include alginate, chitosan, starch, cellulose, collagen, gelatine, and liposome. Synthetic biodegradable polymer materials are mainly composed of polyester, polyamide, and polyurethane. The preparation processes for most synthetic polymer materials produce some residual organic solvents, which may cause damage and result in GF inactivation or aggregation. With this negative impact in mind, a water-soluble polymer (such as alginate or chitosan) may be a better choice for a growth factor delivery system. Alginate, a polysaccharide biopolymer typically derived from seaweed or algae, has been receiving increasing attention. Researchers favour alginate as a scaffold for tissue engineering and as a factor delivery vehicle for the following reasons. First, one of the compelling advantages is that alginate solution quickly crosslinks to form a hydrogel under mild and physiological conditons when exposed to divalent cations [38]. From the perspective of preserving the viablitiy of incorporated proteins, the gentle conditions required for gelling is an advantage that minimises the detrimental effects of the gelling process [39]. Second, the interconnected porous structure formed in the coagulation process allows for nutrient and oxygen diffusion [40,41] and is advantageous for regulating the slow release of bioactive agents including GFs. Because alginate confers so many significant advantages, alginate was adopted as the PRP-derived growth factor release carrier in the present study.

PRP alginate composites, such as PRP alginate beads and PRP alginate capsules, confer several potential advantages over the PRP gel. Most notably, the beads and capsules are superior in terms of cost-effectiveness, sustained delivery, safety, optimised platelet activation methods, and clinical efficiency. The greatest advantage of these composites is that alginate, a sustained release delivery carrier of GFs, was applied in the preparation of PRP alginate composites. Another advantage that cannot be ignored is the difference in platelet activation style between the PRP alginate composite and PRP gel. The 2 most commonly described PRP activation methods in clinical use are (1) using bovine thrombin in combination with calcium chloride, with which the platelet activation of PRP alginate composite is formed, or (2) using calcium chloride alone, with which PRP alginate composite is formed. A relevant report about which activation method would be more effective by Textor et al [42] indicated that the $CaCl_2$ activation of PRP yielded significantly greater growth factor than did any other method, and this method was recommended for clinical use. Textor et al [42] also showed that the process of activation by $CaCl_2$ is slower and more physiologic than that of externally provided thrombin, which immediately initiates a receptor-driven activation process on the platelet. Furthermore, another important advantage is that PRP alginate composites are safer than PRP gel is. Bovine thrombin is a fast and potent method for platelet activation that has been widely used for intraoperative PRP application in humans since 1945 [43]. However, it is important to note that some publications [44-46] still report potential safety risks (i.e., spread of bovine spongiform encephalopathy, potential immune response to a foreign protein, disorders of the body blood coagulation system, and/or nonspecific proteolytic tissue damage) in the clinical application of bovine thrombin, although a majority of studies have not confirmed immunological responses or other adverse reactions induced by bovine thrombin. Therefore, it is assumed that bovine thrombin poses a potential risk and should be avoided if an alternative method for PRP activation is available.

The results presented in this work do not contradict our hypothesis but suggest that the two different types of PRP alginate hydrogel differ in terms of stimulating tissue repair and that PRP alginate beads, as prepared in the present study, are the most effective material for bone formation. Potential explanations for this observation are as follows. First, and most importantly, variations in the gelation process in the preparation of carrier types could have resulted in different outcomes, although the capsules and beads were both composed of PRP and alginate. Algiante beads containing PRP were formed via the internal gelation process, while the PRP alginate capsules were formed via the external gelation process [26]. Helen et al stated that these two distinct processes lead to markedly different exposure times of PRP to alginate [26]. They also demonstrated that the prolonged explosure of PRP to alginate during bead formation permits the PRP-derived factors to readily bind to the polymer matrix and that the rapid crosslinking of the polymer chains by Ca^{2+} during capsule gelation may decrease the availability of PRP-derived growth factor binding domains in alginate [26]. The factor release profiles varied as a function of carrier type, and the regeneration ability thus also varied. Another aspect that should be considered to explain the results observed in the present study is the assumption that the PRP alginate beads had a slower degradation rate than did the PRP alginate capsules. This assumption is based on the results of a study by Sylvia et al [25], which indicated that alginate capsules were more effective in retaining growth factors compared with alginate beads. This property allows the retention of growth factors to be released continuously over a sufficiently long period of time and thus induces greater bone formation with the PRP-alginate capsule treatment than with the PRP-alginate bead treatment. In addition, a possible explanation according to the SEM observations is that the PRP-alginate bead scaffold was superior compared with

those of the PRP-alginate capsule and the PRP gel in terms of the surface morphology and adsorption capability of platelet. Considering these factors, we conclude that PRP alginate beads are likely a valuable therapeutic material for rabbits and humans compared with PRP alginate capsules, particularly in open surgical applications.

Based on these in vivo data and their ease of use, PRP alginate beads as tissue engineering scaffolds are a strongly recommended biomaterial for regeneration medicine. However, a variety of problems remain to be solved, such as whether the structure and mechanical properties of new bone tissue meet the physical requirements and whether the final outcome of the cell carrier complex is ideal. Consequently, we believe that it is necessary to further modify and optimise this biomaterial. Meanwhile, the therapeutic and regenerative effectiveness of these biomaterials still require testing through human clinical experiments. This tissue engineering technique still has a long way to progress before reaching clinical feasibility.

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