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Original Article

Effect of leukocyte and platelet-rich plasma on osseointegration after implant placement in mouse maxilla

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ABSTRACT

Introduction: Osseointegration, the direct contact between an implant and bone, can be achieved by direct and/or indirect osteogenesis. Platelet-rich plasma accelerates tissue regeneration, wound healing, and osseointegration. This study aimed to analyze the effects of leukocyte and platelet-rich plasma (L-PRP) on direct and indirect osteogenesis after implant placement in a mouse maxilla.
Methods: Blood was collected from the tail vein of 4–8-week-old male ICR mice and L-PRP was obtained after double-spin cycle centrifugation. After the right upper first molars of 4-week-old ICR mice were extracted while under deep anesthesia, the alveolar sockets were prepared with a drill, and titanium implants blasted with hydroxyapatite/β-tricalcium phosphate were placed into the cavity filled with 1.5 μL of L-PRP. Samples were collected from the animals 3–28 days after implantation, and immunohistochemistry for osteopontin, Ki67 (cell proliferation marker), cathepsin-K (osteoclast marker), and osteonectin (osteoblast marker) was performed.
Results: Cell proliferation was significantly higher in the L-PRP group than in the control group on postoperative days 3 and 5. The activities of osteoclast-lineage cells and osteoblasts increased significantly on day 5 in the L-PRP group, indicating that L-PRP evoked an active cellular response. Indirect osteogenesis was significantly higher on days 7, 14, and 28, and the osseointegration rate was significantly higher on day 28 in the L-PRP group compared with the control group.
Conclusions: L-PRP enhances osseointegration by promoting mesenchymal cell proliferation, osteoclastic and osteoblastic activities, and indirect osteogenesis.

1. Introduction

Osseointegration, which is defined as the direct contact of living bone with the surface of a load-bearing dental implant at a light microscope level [1], is a crucial factor in controlling the fate of the implant. It is also defined according to Miller et al. as the formation of a direct interface between living pre-existing bone and implant without the soft tissue [2]. After implant placement, the space between the pre-existing bone and implant surface is filled with blood, and a blood clot, including its fibrin network, is subsequently formed around the implant with the infiltration of neutrophils and macrophages, which is then replaced with granulation tissue, leading to bone formation [3]. Wound healing around an endosseous implant in a mouse model is similar to that in humans, although the speed and mode of healing differ between the two species. The inflammatory phase occurs on days 1–3; the proliferative phase on

Abbreviations: PRP, platelet-rich plasma; LPRP, leukocyte and platelet-rich plasma; PRF, platelet-rich fibrin; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor-β1; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; ICR, institute of cancer research; PFA, paraformaldehyde; H&E, hematoxylin and eosin; OPN, osteopontin.

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days 5–7; and the remodeling phase on weeks 2–4 [4]. Osseointegration can be divided into two modes. Osteoblasts may deposit bone matrix on the bone surface toward the implant following active bone resorption by osteoclasts; this mode is known as “distance” or “indirect” osteogenesis [5–7]. In contrast, osteoclast-lineage cells may be recruited to the implant surface and induce osteoblast differentiation on the implant, which results in bone formation from the implant surface to the bone; this mode is referred to as “contact” or “direct” osteogenesis [5–7]. When bone consisting of a woven bone matrix achieves a certain thickness, lamellar bone formation begins [1]. The simultaneous occurrence of both types of osteogenesis is advantageous for increased implant stability, thereby improving osseointegration [5].

Platelet-rich plasma (PRP) has been used in the dental field since the early 1990s for various applications to accelerate tissue regeneration, improve wound healing, and promote osseointegration [8,9]. PRP is a biological product that originates from the plasma fraction of autologous blood with a supraphysiological level of platelets, collected from patients before centrifugation [8,10,11]. It is an adjuvant therapy, the mechanism of which depends on successful angiogenesis [12]. The first study considering the clinical benefits of PRP regarding osseointegration after dental implantation was reported in 1998 by Marx et al., who described an enhancing effect of PRP on the density of bone grafts in mandibular defects [13]; specifically, PRP therapy significantly increased trabecular bone density [14]. Systematic reviews and meta-analyses have provided evidence supporting the clinical benefits of PRP on bone regeneration and osseointegration, where PRP and platelet-rich fibrin (PRF) promote the initial osseointegration process and increase implant stability [15,16]. In addition, several experimental models have demonstrated the positive effects of PRP on promoting bone regeneration in bone defects, bone healing, and osseointegration [10,11,17]. However, the majority of human and animal studies have focused on 2D and 3D microfocus computed tomography (μ-CT) imageology but failed to clarify the chronological cellular events including cell proliferation and differentiation using cell differentiation markers [10,18,19]. Leukocyte and platelet-rich plasma (L-PRP) is a platelet concentrate with leukocytes, being characterized by a low-density fibrin network after activation [20], which makes the application technique (injection) easier and allow to obtain larger volume of final product with the same amount of whole blood. Furthermore, L-PRP promotes bone regeneration [11] and is more effective for controlling postoperative infections at surgical sites [21–23]. L-PRP antimicrobial effects against *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* have been reported in the previous studies [24,25].

This study aimed to evaluate the beneficial effects of leukocyte and platelet-rich plasma (L-PRP) on osseointegration after immediate implant placement in the mouse maxilla and provide further evidence regarding this contentious topic.

2. Methods

2.1. Animals and experimental procedures

All the animal experiments were performed according to the protocol that was reviewed by the Institutional Animal Care and Use Committee and approved by the President of Niigata University (SA01338). Male Crlj:CD1 Institute of Cancer Research (ICR) mice (four weeks old) were obtained from Charles River Laboratories Japan (Yokohama, Japan). ICR mice is most commonly available outbred population because they have good reproductive performance, are inexpensive, robust, and grow rapidly, being excellent models in a wide range of research fields. Their genetic variability provides an accurate representation of the genetic diversity found in the human model [26–28]. Blood was collected from the tail vein of 4–8-week-old mice to prepare L-PRP, which was selected because of its content, including active leukocytes against selected bacterial strains, and its characteristics of a low-density fibrin network after activation. In addition, it is widely used in orthopedic, maxillofacial, and cardiac surgical procedures [8,20,24] as well as a large number of commercial and experimental systems [24]. The L-PRP was prepared via double-spin cycle centrifugation (Kubota 3500 and 4000, Tokyo, Japan). The first spin consisted of a horizontal spin of 400×*g* for 10 min, the supernatant was removed (platelet-poor plasma), and 1 μL of prostaglandin E1 was added to reduce platelet activation. The second spin was centrifuged at 900×*g* for 3.5 min. After the second spin, 0.1 mL of the L-PRP was analyzed in a Blood Cell Counting Device (pocH iV-diff, Sysmex Corporation, Kobe, Japan) to determine the final blood cell concentration. An amount of 0.2 mL of L-PRP was determined for use in subsequent experiments (Supplementary Fig. 1).

2.2. Implant placement

All surgeries were performed under anesthesia using an intraperitoneal injection of combined solution (0.05–0.1 mL/10 g) of 1.875 mL Domitor® (Nippon Zenyaku Kogyo Co, Ltd., Koriyama, Japan), 2 mL of midazolam (Sandoz KK, Tokyo, Japan), 2.5 mL of Vetorphale® (Meiji Seika Pharma Co, Ltd., Tokyo, Japan), and 18.625 mL of physiological saline. The right first maxillary molars from 4-week-old mice were extracted with a pair of modified dental forceps, and the socket was prepared with a drill (the diameter and depth of the cavity were 1 and < 2 mm, respectively). A titanium implant blasted with

Table 1
Quality of leukocyte and platelet-rich plasma including platelet and white blood cell concentration. The number of mice designed for experiments in each operation is displayed in the “Target subjects” column and the observation period is indicated in parenthesis.

Operation	Mice age	Mice number	Platelets in whole blood ($x10^4$/μL)	Platelets in PRP ($x10^4$/μL)	Times-fold increase	White blood cell concentration ($x10^2$/μL)	Target subjects
1	4 weeks	6	55.4	87.2	1,6x	21	5 mice (7 days)
2	8 weeks	6	85.8	384.5	4.5x	138	6 mice (7 days)
3	4 weeks	6	78.8	431.8	5.5x	52	14 mice (14 days)
4	6 weeks	6	100.4	941.4	9.4x	172	11 mice (3 days) 10 mice (5 days)
5	4 weeks	6	97.5	418.3	4.3x	103	4 mice (7 days) 9 mice (28 days)

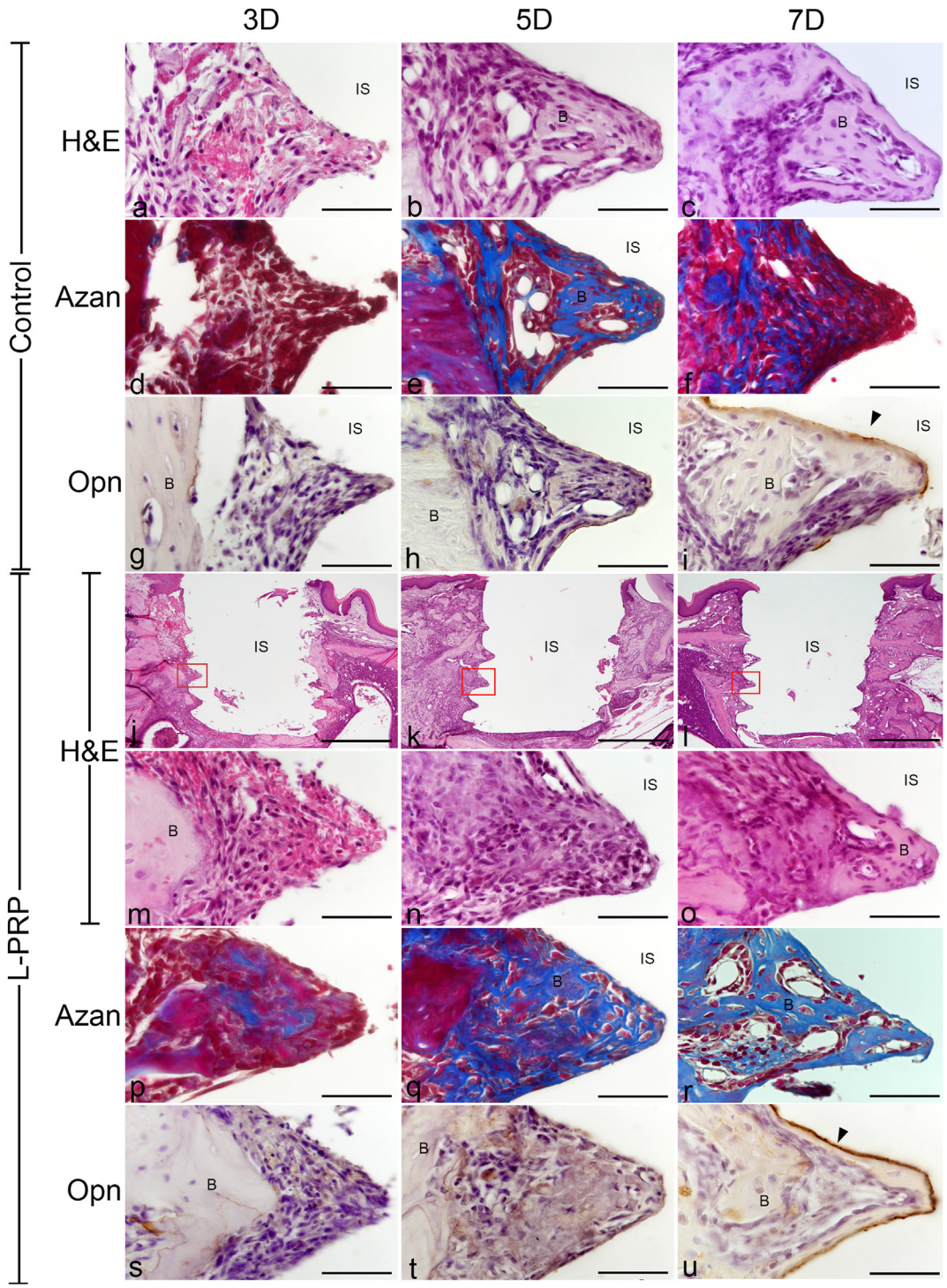

Fig. 1. Hematoxylin & eosin (H&E)-staining (a–c, j–o), Azan-staining (d–f, p–r) and osteopontin (OPN)-immunoreactivity (g–i, s–u) in the tissues surrounding the implants on day 3 (a, d, g, j, m, p, s), 5 (b, e, h, k, n, q, t), and 7 (c, f, i, l, o, r, u) after implant placement. (a, d, j, m, p) On day 3, the surrounding tissues are occupied by inflammatory cells, fibroblasts, red blood cells, and eosinophilic matrices. Figure m is a higher magnification of the boxed area in Figure j. (g, s) OPN deposition is observed in the pre-existing bone. (b, e, k, n, q) On day 5, the inflammatory reaction is weaker, clear blood vessel lumens are visible, and some areas are filled with new bone matrices. Figure n is a higher magnification of the boxed area in Figure k. (h, t) A weak OPN-positive immunoreaction is noticeable in areas with new bone formation. (c, f, l, o, r) On day 7, direct and indirect osteogenesis occur at the implant-bone interface. Figure o is a higher magnification of the boxed area in Figure l. (i, u) There is a strong OPN-positive immunoreaction at the interface (arrowheads). B, bone; IS, implant space. Scale bars: 500 μm (j–l) and 50 μm (a–i, m–u).

hydroxyapatite/β-tricalcium phosphate (Prosper, Kashiwazaki, Japan) was inserted into the cavity using a screwdriver after the bleeding was controlled. An amount of 1.5 μL of L-PRP was injected into the alveolar cavity before the implant placement for the L-PRP group.

2.3. Histological procedure

Mice from the L-PRP and control groups were sacrificed on days 3, 5, 7, 14, and 28 after implantation. At each time point, the mice were perfused with 4% paraformaldehyde (PFA) (Fujifilm Wako;

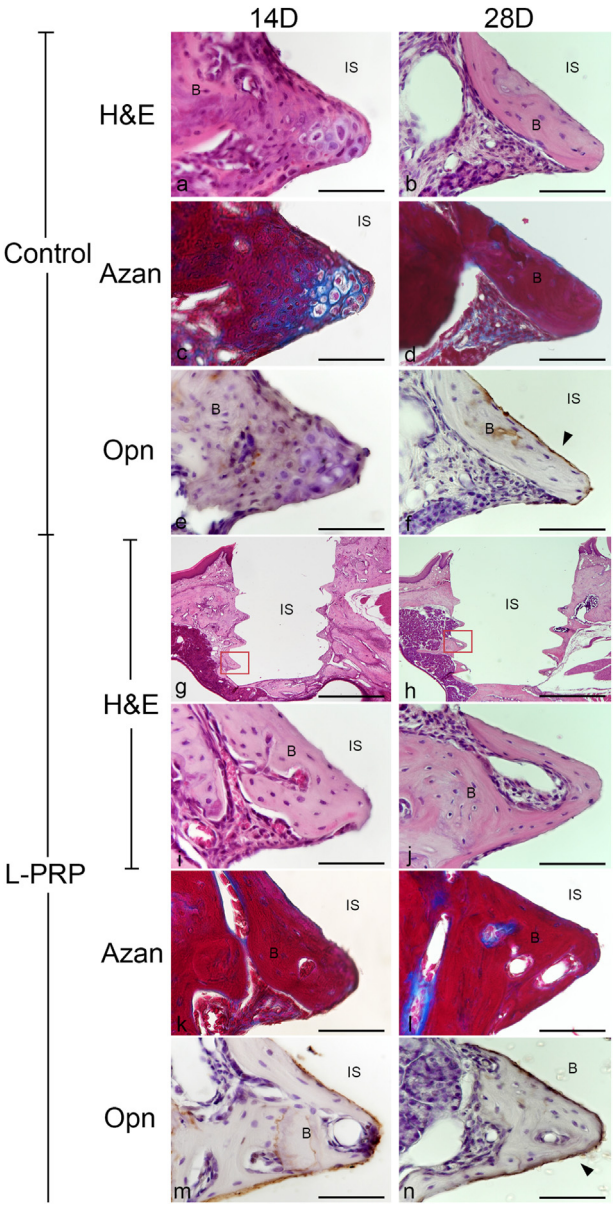

Fig. 2. H&E-staining (a, b, g–j), Azan-staining (c, d, k, l), and OPN-immunoreactivity (e, f, m, n) in the tissues surrounding the implants on days 14 (a, c, e, g, i, k, m) and 28 (b, d, f, h, j, l, n) after implant placement. (a, c, g, i, k) On day 14, new mature bone with typical osteoblasts and osteocytes is observed in the leukocyte and platelet-rich plasma (L-PRP) group. Figure i is a higher magnification of the boxed area in Figure g. (e, m) A clear OPN-positive immunoreaction is observed in the L-PRP group. (b, d, h, j, l) On day 28, mature bone is evident even in the control group. Figure j is a higher magnification of the boxed area in Figure h. (f, n) The OPN-positive immunoreaction is stronger in the L-PRP group (arrowheads). B, bone; IS, implant space. Scale bars: 500 μm (g, h) and 50 μm (a–f, i–n).

Osaka, Japan; CAS RN: 30525-89-4) in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia with an intraperitoneal injection of a combined solution of Domitor®, midazolam, Vetorphale®, and physiological saline. The maxillae were removed and immersed in 4% PFA for 24 h. After decalcification in Morse's solution at 4 °C, the samples were dehydrated with a series of ethanol and embedded in paraffin after the implant removal. Then 4-μm sagittal sections were obtained for hematoxylin & eosin (H&E) and Azan staining, and immunohistochemistry for osteopontin (OPN), Ki67, osteonectin, and cathepsin-K.

2.4. Immunohistochemical analyses

Immunohistochemistry was performed using a rabbit anti-OPN polyclonal antibody diluted to 1:5000 (LMS Co., Ltd., Tokyo, Japan; catalog no. LSL-LB-4225), a mouse anti-Ki67 monoclonal antibody diluted to 1:100 (Dako, Tokyo, Japan; catalog no. M7249) for the cell proliferation assay, a mouse anti-cathepsin K diluted to 1:200 (Daiichi Fine Chemical Co., Ltd., Japan; No. F-95, Clone No. 182-12G5) for the positive area assay of osteoclast-lineage cells and a rabbit anti-osteonectin polyclonal antibody diluted to 1:800 (LMS Co., Ltd., Tokyo, Japan; catalog no. LSL-LB-4115) for the positive area assay of osteoblasts beneath the bone matrix. This was perfumed using the Envision+/horseradish peroxidase system (Dako; catalog no. K5027) and the avidin-biotin peroxidase complex (Vectastain ABC Kit, Vector Laboratories) method with a biotinylated anti-mouse IgG (H + L) diluted to 1:100 (Vector Laboratories, USA; catalog no. BA-2001) for Ki67 and cathepsin K and a biotinylated anti-rabbit IgG (H + L) diluted to 1:400 (Vector Laboratories, USA; catalog no. BA-1000) for osteonectin. To visualize of the sections, 0.05 M Tris-HCl buffer (pH 7.6) containing 0.04% 3-3′-diaminobenzidine tetrahydrochloride (Dojindo; Japan; Code: D006 DAB) and 30% H_2O_2 were used. Counter-staining was performed with hematoxylin.

2.5. Statistical analysis

The OPN-positive perimeter and cell proliferation assays were conducted using ImageJ software 1.53 (National Institutes of Health, Bethesda, MD, USA). The percentage of Ki67-positive cells at the bone-implant interface of each specimen was obtained by using a grid of 208 × 159 μm^2. The osseointegration rate was analyzed in the H&E sections using Photoshop 2023 (Adobe Inc., San Jose, CA, USA) by measuring the direct and indirect osteogenesis. This is done by obtaining the whole perimeter of the implant surface and measuring the areas where new bone is in direct contact with the implant space. Cathepsin K and osteonectin in the samples were analyzed using WinROOF software (ver 7.4.0; 2013 Mitani, Japan) to obtain the percentage of positive areas per the whole area of the surrounding tissues. To determine the osteonectin-positive area, the positive cells beneath the bone matrix were exclusively selected; cells entrapped in the bone matrix (osteocytes) were excluded. Statistical analysis was performed using IBM® SPSS® (Ver 21, IBM, Tokyo, Japan). The normality of the data was analyzed using the Shapiro–Wilk test. For the comparison between the L-PRP and control groups, the Student's *t*-test was conducted after the confirmation of data normality and homogeneity of variance. For chronological changes, the Bonferroni test for multiple comparisons was used. The rate of OPN-positive perimeter at each time point after implantation was compared using one-way analysis of variance after confirming data normality and homogeneity of variance, followed by the Bonferroni test for multiple comparisons. The samples that exhibited no normal distribution were compared using the Kruskal–Wallis test for three or more groups or Mann–Whitney *U* test for two groups. The data were reported as the mean ± standard deviation (SD).

3. Results

3.1. L-PRP quality

The data on L-PRP quality, including platelet and white blood cell concentration, are shown in Table 1. The highest concentration of platelets was obtained from 6-week-old mice, which were used

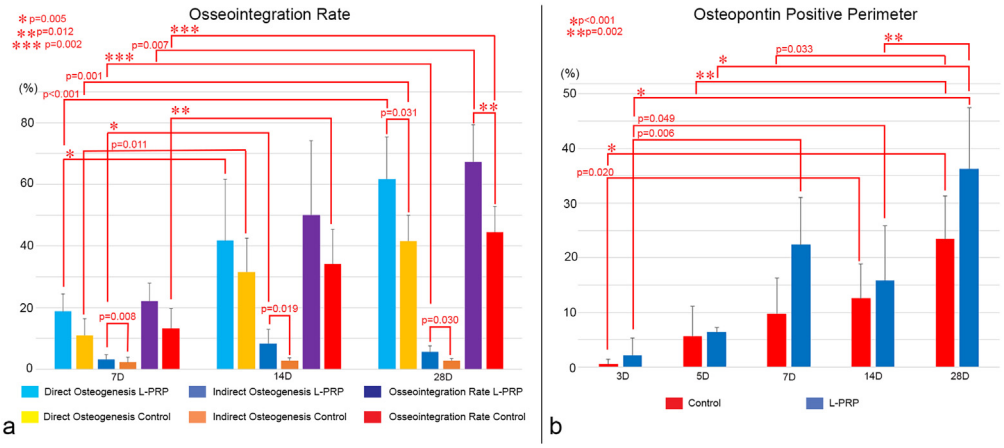

Fig. 3. Chronological changes of the rate of osseointegration, direct and indirect osteogenesis (a), and the OPN-positive perimeter (b) between the control and L-PRP groups. (a) There is a significant difference in the osseointegration rate at day 28 and the indirect osteogenesis on days 7, 14 and 28. (b) The OPN-positive perimeter around the implant surface significantly increases in the L-PRP group rather compared with the control group. The values represent the mean ± standard deviation (SD).

for providing blood for the mice fixed 3 and 5 days after implant placement.

3.2. Osseointegration and OPN-positive rates

On day 3, the surrounding tissues were occupied by inflammatory cells, fibroblasts, red blood cells, and eosinophilic matrices in the control and L-PRP groups. No bone formation occurred in either group. The cells and eosinophilic matrices in the L-PRP group occupied the implant-bone interface more densely compared with the control group, showing artificial shrinkage (Fig. 1a, d, j, m, p). OPN deposition was observed in the pre-existing bone of the control and L-PRP groups (Fig. 1g, s). On day 5, the inflammatory reaction became weaker and the clear lumens of blood vessels were visible, revealing the establishment of revascularization. Some areas were filled with new bone matrices secreted by osteoblasts (Fig. 1b, e, k, n, q). A weak OPN-positive immunoreaction was identifiable in the areas with new bone formation (Fig. 1h, t). On day 7, direct and indirect osteogenesis occurred in the implant-bone interface in both the control and L-PRP groups (Fig. 1c, f, l, o, r). There were strong OPN-positive immunoreactions at the surface of the implant where direct osteogenesis occurred in both groups (Fig. 1i, u). On day 14, new mature bone with typical osteoblasts and osteocytes was observed in the L-PRP group (Fig. 2a, c, g, i, k). On day 28, mature bone with the presence of osteocytes and osteoblasts was present in both groups; however, it covered a great perimeter in the L-PRP group (Fig. 2b, d, h, j, l). A significant difference was found in the osseointegration rate on day 28 days after implant placement in the L-PRP group (Fig. 3a). Indirect osteogenesis was significantly increased in the L-PRP group on days 7, 14, and 28 postoperation (Fig. 3a). A clear OPN-positive immunoreaction was observed in the L-PRP group (Fig. 2e, m). On day 28, mature bone was evident even in the control group. The OPN-positive immunoreactivity was stronger in the L-PRP group, compared with the control group (Fig. 2f, n). The OPN-positive perimeter around the implant surface revealed a tendency to increase in the L-PRP group compared with the control group (Fig. 3b).

3.3. Osteoclast-lineage cell and osteoblast activity

On day 3, cathepsin K-positive osteoclast-lineage cells surrounding the pre-existing bone were detectable in both the control and L-PRP groups. No bone formation was observed around the implant surface in both groups (Fig. 4a–d). On day 5, the expression of cathepsin K immunoreactivity was stronger in the L-PRP group; however, no significant difference was found. The presence of osteonectin-positive osteoblasts and cathepsin K-positive osteoblasts surrounding the newly formed bone matrix was observed (Fig. 4e–h). The cathepsin K and osteonectin immunoreactivity reached a peak on day 5 in the L-PRP group. There was a significant increase in the number of osteoclast-lineage cells and osteoblasts at this time point (Fig. 4u and v). On day 7, the immunoreactivity of osteoclast-lineage cells was lower than that of day 5. The osteoblasts aligned around the newly formed bone matrix, with some getting trapped in the bone matrix and becoming osteocytes (Fig. 4i–l). On days 14 and 28, the control group exhibited a higher trend of osteonectin-positive osteoblasts compared with the L-PRP group. The osteoclast-lineage cells and osteoblasts remained around the blood vessels (Fig. 4m–t).

3.4. Cell proliferation

On day 3, cell proliferation was higher in the L-PRP group than in the control group, which was the peak of cell proliferation (Fig. 5a and b). On day 5, cell proliferation remained higher in the L-PRP group compared with the control group (Fig. 5c and d). On day 7, cell proliferation decreased in the L-PRP group (Fig. 5e and f). On days 14 and 28, proliferative activity ceased in the control and L-PRP groups (Fig. 5g–j). There were significant differences between the control and L-PRP groups on days 3 and 5, and the proliferative activity in the L-PRP group significantly decreased on days 7–28 (Fig. 5k).

4. Discussion

This study demonstrated that osseointegration was significantly improved in the L-PRP group 28 days after implant placement, where the rate of osseointegration reached 67%, and that indirect osteogenesis was significantly enhanced during days 7–28 postoperation. These findings are supported by previous studies that reported a higher bone regeneration rate in human participants or in rabbits using platelet concentrates [29–35]. In contrast, several studies using platelet concentrates failed to demonstrate the decisive superiority of their use with immediate implant placement regarding osseointegration [36]. Direct osteogenesis is directly related to the presence of OPN on the dental implant surface [7]. In

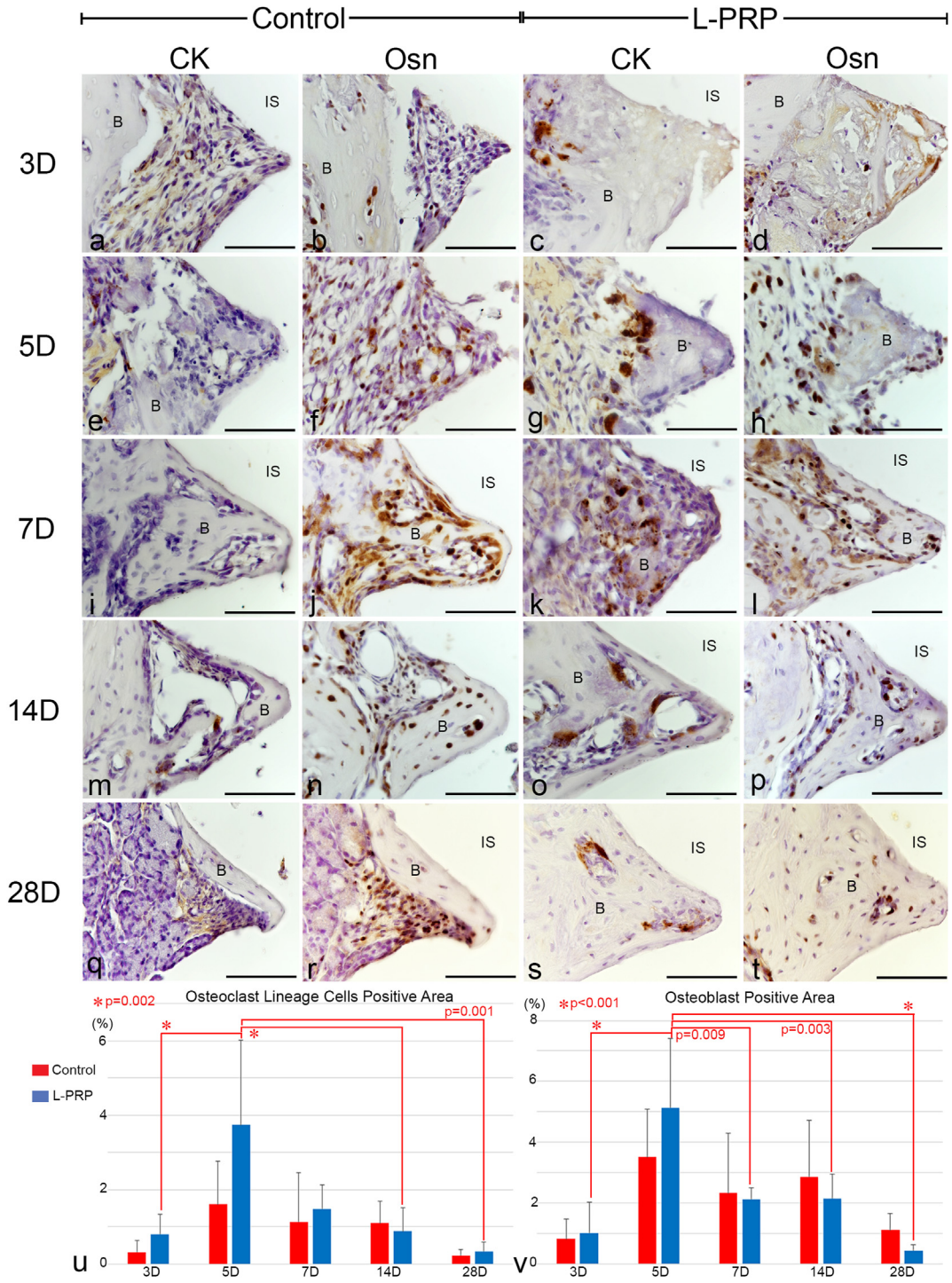

Fig. 4. Cathepsin-K (a, c, e, g, i, k, m, o, q, s) and osteonectin (b, d, f, h, j, l, n, p, r, t) immunoreactivity in the surrounding tissues on days 3 (a–d), 5 (e–h), 7 (i–l), 14 (m–p), and 28 (q–t) after implant placement. (a–d) On day 3, cathepsin K-positive osteoclast-lineage cells surrounding the pre-existing bone are detectable in the control and L-PRP groups. However, no bone formation is observed around the implant surface. (e–h) On day 5, the expression of cathepsin K immunoreactivity is stronger in the L-PRP group than in the control group. Osteonectin-positive osteoblasts and cathepsin K-positive osteoclasts surrounding the newly formed bone matrix are noticeable. (i–l) Cathepsin-K immunoreactivity on day 7 is lower than that on day 5. The osteoblasts align around the newly formed bone, with some getting trapped in the bone matrix and becoming osteocytes. (m–t) During days 14–28, the control group shows a higher trend of osteonectin-positive osteoblasts compared with the L-PRP group. The osteoclast-lineage cells and osteoblasts remain around the blood vessels. (u, v) The number of osteoclast-lineage cells and osteoblasts reach a peak on day 5. There is a significant increase and decrease of osteoclast-lineage cells and osteoblasts at this time point, respectively. B, bone; IS, implant space. Scale bars: 50 μm.

this study, the highest percentage of OPN-positive perimeter and direct osteogenesis was obtained 28 days after implant placement; however, no significant difference was observed between the control and L-PRP groups. Interestingly, indirect osteogenesis in the L-PRP group significantly increased during days 7–28 compared with the control group. This could be explained by the difference in micro circumstances between the implant surface with no cellular components and the pre-existing bone surface with osteoblast-

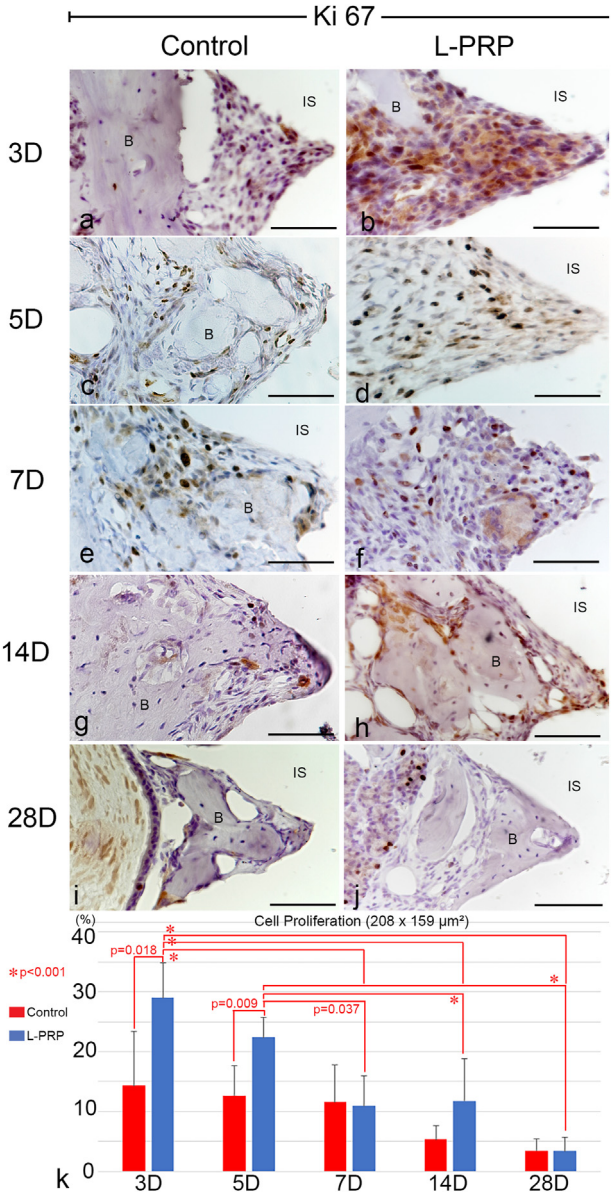

Fig. 5. Ki67-immunoreactivities in the surrounding tissues (a–j) and the rate of cell proliferation on day 3 (a, b), 5 (c, d), 7 (e, f), 14 (g, h), and 28 (i, j) after implant placement in the control (a, c, e, g, i) and L-PRP groups (b, d, f, h, j). (a–b) On day 3, cell proliferation is high in the L-PRP group. (c, d) On day 5, cell proliferation remains high in the L-PRP group, but (e, f) on day 7, cell proliferation decreases. (g–j) On days 14 and 28, cell proliferation almost ceases. (k) There are significant differences between the control and L-PRP groups on days 3 and 5 after implant placement. B, bone; IS, implant space. Scale bars: 50 μm.

lineage cells. Growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor β1 (TGF-β1) that are contained in the L-PRP, are bound to the receptors expressed on the osteoblast cell membranes that have aligned on the pre-existing bone surface [22] and have a strong chemotactic effect on mesenchymal cells [30]. As no matrix is present on the surface of an endosseous implant, the surface must be colonized by osteoclast-lineage cells followed by osteoblasts before bone matrix deposition [37]. This is the main reason why indirect osteogenesis requires a blood supply and growth factors immediately before the production of an osteoid matrix [14]. As a result, osseointegration was enhanced by the significant improvement of indirect osteogenesis because of the growth factors in the L-PRP.

Cell proliferation significantly increased in the L-PRP group on days 3 and 5, with its highest rate observed on day 3. This is supported by previous studies using a mouse model without the use of PRP, where cell proliferation reached a peak 3 days after implant placement [4,7]. The growth factors present in PRP play an important role in cell proliferation and differentiation. Growth factors promote the proliferation of bone marrow-derived mesenchymal stem cells that have the potential to differentiate into osteoblasts [38]. Therefore, proliferation activity was increased by the addition of L-PRP during the early stages and began to decrease after day 5 because the differentiation of osteonectin-positive osteoblasts began on day 5. After day 5, there was a significant increase in the activity of osteoclast-lineage cells and osteoblasts in the L-PRP group. These findings suggest the promotion of bone resorption and formation with strong osteogenic cell activity by L-PRP treatment. An *in vitro* study evaluated the effect of PRP on osteoblasts and fibroblasts and demonstrated an increased production of osteoblasts with PRP treatment after 72 h of cell culture [39]. The positive effect of PRP on osteoblasts was confirmed by a previous *in vivo* experiment using rabbits [33], which revealed that osteoblasts in the osteoid matrix were positive for PDGF and TGF-β1 receptors [33,37]. The effect of growth factors on cell proliferation, osteoblastic differentiation, and osteoclastogenesis is dose-dependent because very high concentrations thereof can increase cell differentiation instead of cell proliferation [22]. High concentrations result in pH changes that negatively affect cell proliferation [40]. In addition, the overexpression of TGF-β1 promotes osteogenic differentiation and osteoclastogenesis during the early stages of bone healing [22]. However, no significant difference was found in this study when different platelet concentrations were compared on day 7. Thus, the growth factors in L-PRP stimulate an increase in the activity of osteogenic cells, leading to bone resorption and formation.

To the best of our knowledge, this is the first study to evaluate the enhancement of osseointegration using PRP in a mouse model with cellular level analyses including chronological changes with immunohistochemical markers. Furthermore, many previous studies regarding platelet concentrates and osseointegration failed to demonstrate the cell concentration used in their experiments. This could lead to bias because optimal platelet concentrations can promote cell proliferation, angiogenesis, and osteogenic precursor differentiation [38]. In addition, it is important to standardize the preparation protocols for platelet concentrates. The existence of many protocols is attributed to the diversity of PRP and PRF systems and the different types of platelet concentrates regarding their cell content and therapeutic application [22]. Thus, the standardization of the preparation protocol and the optimal analyses of the platelet concentration are the next challenges. Another limitation is that this study focuses on *in vivo* histological study and fails to clarify the mechanism of the obtained results with *in vitro* analyses. Further studies should attempt to standardize the animal model and preparation protocol based on histomorphometric and immunohistochemical studies as well as *in vitro* approaches to support the current findings. Furthermore, there is a need for long-term, large-scale prospective studies to demonstrate the effectiveness of PRP with regard to osseointegration in an animal model [16,21,34].

The animal experimental model using mice is useful for the evaluation of histological responses to implant materials and provides highly reproducible experiments because murine wound healing around an endosseous implant is similar to that in humans and faster, achieving bone remodeling in 2–4 weeks [4,5]. The *in vivo* experimental studies using mice allow the transfer of the results in mice into the clinical practice in the human aiming to accelerate the process of bone formation and osseointegration around dental implants [10].

5. Conclusions

Osseointegration and indirect osteogenesis were enhanced by the application of L-PRP 28 days after implant placement by promoting cell proliferation during the early stages as well as osteoblastic and osteoclastic activity 5 days postoperation. Future research should attempt to standardize the L-PRP preparation protocol focusing on long-term, large-scale prospective studies. Furthermore, *in vitro* studies are needed for clarifying the mechanism of the obtained results.

Declaration of competing interest

The authors declare no conflicts of interest related to this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.08.019.

References

[1] Adell R, Lekholm U, Rockler B, Branemark PI. A 15-year study of osseointegrated implants in the treatment of the edentulous jaw. Int J Oral Surg 1981;10:387–416.
[2] Miller BF, Keane CB. Miller-Keane Encyclopedia & dictionary of medicine, nursing & allied health. sixth ed. Philadelphia: Saunders; 1997.
[3] Li J, Jansen JA, Walboomers XF, van den Beucken JJ. Mechanical aspects of dental implants and osseointegration: a narrative review. J Mech Behav Biomed Mater 2020;103:103574.
[4] Watanabe T, Nakagawa E, Saito K, Ohshima H. Differences in healing patterns of the bone-implant interface between immediately and delayed-placed titanium implants in mouse maxillae. Clin Implant Dent Relat Res 2016;18:146–60.
[5] Makishi S, Saito K, Ohshima H. Osteopontin-deficiency disturbs direct osteogenesis in the process of achieving osseointegration following immediate placement of endosseous implants. Clin Implant Dent Relat Res 2017;19:496–504.
[6] Makishi S, Watanabe T, Saito K, Ohshima H. Effect of hydroxyapatite/beta-tricalcium phosphate on osseointegration after implantation into mouse maxilla. Int J Mol Sci 2023;24.
[7] Makishi S, Yamazaki T, Ohshima H. Osteopontin on the dental implant surface promotes direct osteogenesis in osseointegration. Int J Mol Sci 2022;23.
[8] Alves R, Grimalt R. A review of platelet-rich plasma: history, biology, mechanism of action, and classification. Skin Appendage Disord 2018;4:18–24.
[9] Stahli A, Strauss FJ, Gruber R. The use of platelet-rich plasma to enhance the outcomes of implant therapy: a systematic review. Clin Oral Implants Res 2018;29(Suppl 18):20–36.
[10] Stramandinoli-Zanicotti RT, Sassi LM, Rebelatto CL, Boldrine-Leite LM, Brofman PR, Carvalho AL. The effect of bone marrow-derived stem cells associated with platelet-rich plasma on the osseointegration of immediately placed implants. J Clin Exp Dent 2021;13:e8–13.
[11] Yin W, Qi X, Zhang Y, Sheng J, Xu Z, Tao S, Xie X, Li X, Zhang C. Advantages of pure platelet-rich plasma compared with leukocyte- and platelet-rich plasma in promoting repair of bone defects. J Transl Med 2016;14:73.
[12] Takahashi A, Takahashi S, Tsujino T, Isobe K, Watanabe T, Kitamura Y, Watanabe T, Nakata K, Kawase T. Platelet adhesion on commercially pure titanium plates in vitro I: effects of plasma components and involvement of the von Willebrand factor and fibronectin. Int J Implant Dent 2019;5:5.
[13] Roffi A, Filardo G, Kon E, Marcacci M. Does PRP enhance bone integration with grafts, graft substitutes, or implants? A systematic review. BMC Muscoskel Disord 2013;14:330.
[14] Kim UG, Choi JY, Lee JB, Yeo IL. Platelet-rich plasma alone is unable to trigger contact osteogenesis on titanium implant surfaces. Int J Implant Dent 2022;8:25.
[15] Gonzalez-Serrano J, Vallina C, Gonzalez-Serrano C, Sanchez-Monescillo A, Torres J, Hernandez G, Lopez-Pintor RM. Effect of platelet concentrates on marginal bone loss of immediate implant procedures: a systematic review and meta-analysis. Materials 2021;14.
[16] Qu C, Luo F, Hong G, Wan Q. Effects of platelet concentrates on implant stability and marginal bone loss: a systematic review and meta-analysis. BMC Oral Health 2021;21:579.
[17] Gopinath A, Ravikanth A, Kadiyala KK, Thota KM, Manne P, Babu MS. Effect of platelet rich plasma on stability of dental implants: a prospective comparative clinical study. J Dr NTR Univ Health Sci 2017;6:107–13.
[18] Naeimi Darestani M, Asl Roosta H, Mosaddad SA, Yaghoubee S. The effect of leukocyte- and platelet-rich fibrin on the bone loss and primary stability of implants placed in posterior maxilla: a randomized clinical trial. Int J Implant Dent 2023;9:23.
[19] Benalcazar Jalkh EB, Tovar N, Arbex L, Kurgansky G, Torroni A, Gil LF, Wall B, Kohanbash K, Bonfante EA, Coelho PG, Witek L. Effect of leukocyte-platelet-rich fibrin in bone healing around dental implants placed in conventional and wide osteotomy sites: a pre-clinical study. J Biomed Mater Res B Appl Biomater 2022;110:2705–13.
[20] Dohan Ehrenfest DM, Andia I, Zumstein MA, Zhang CQ, Pinto NR, Bielecki T. Classification of platelet concentrates (Platelet-Rich Plasma-PRP, Platelet-Rich Fibrin-PRF) for topical and infiltrative use in orthopedic and sports medicine: current consensus, clinical implications and perspectives. Muscles Ligaments Tendons J 2014;4:3–9.
[21] Huang Y, Bornstein MM, Lambrichts I, Yu HY, Politis C, Jacobs R. Platelet-rich plasma for regeneration of neural feedback pathways around dental implants: a concise review and outlook on future possibilities. Int J Oral Sci 2017;9:1–9.
[22] Collins T, Alexander D, Barkatali B. Platelet-rich plasma: a narrative review. EFORT Open Rev 2021;6:225–35.
[23] Khan AS, Zaheer N, Zaigham AM, Shahbaz M, Zaheer U, Alam MK. Effect of platelet-rich plasma on bone healing in immediate implants analyzed by cone beam computerized tomography: a randomized controlled trial. BioMed Res Int 2021;2021:6685991.
[24] Cieslik-Bielecka A, Bold T, Ziolkowski G, Pierchala M, Krolikowska A, Reichert P. Antibacterial activity of leukocyte- and platelet-rich plasma: an in vitro study. BioMed Res Int 2018;2018:9471723.
[25] Mariani E, Canella V, Berlingeri A, Bielli A, Cattini L, Landini MP, Kon E, Marcacci M, Di Matteo B, Filardo G. Leukocyte presence does not increase microbicidal activity of Platelet-rich Plasma in vitro. BMC Microbiol 2015;15:149.
[26] Kim JE, Nam JH, Cho JY, Kim KS, Hwang DY. Annual tendency of research papers used ICR mice as experimental animals in biomedical research fields. Lab Anim Res 2017;33:171–8.
[27] Chia R, Achilli F, Festing MF, Fisher EM. The origins and uses of mouse outbred stocks. Nat Genet 2005;37:1181–6.
[28] Cui S, Chesson C, Hope R. Genetic variation within and between strains of outbred Swiss mice. Lab Anim 1993;27:116–23.
[29] Oncu E, Alaaddinoglu EE. The effect of platelet-rich fibrin on implant stability. Int J Oral Maxillofac Implants 2015;30:578–82.
[30] Tabrizi R, Arabion H, Karagah T. Does platelet-rich fibrin increase the stability of implants in the posterior of the maxilla? A split-mouth randomized clinical trial. Int J Oral Maxillofac Surg 2018;47:672–5.
[31] Boora P, Rathee M, Bhoria M. Effect of platelet rich fibrin (PRF) on peri-implant soft tissue and crestal bone in one-stage implant placement: a randomized controlled trial. J Clin Diagn Res 2015;9:ZC18–21.
[32] ArRejaie A, Al-Harbi F, Alagl AS, Hassan KS. Platelet-rich plasma gel combined with bovine-derived xenograft for the treatment of dehiscence around immediately placed conventionally loaded dental implants in humans: cone beam computed tomography and three-dimensional image evaluation. Int J Oral Maxillofac Implants 2016;31:431–8.
[33] Ortolani E, Guerriero M, Coli A, Di Giannuario A, Minniti G, Polimeni A. Effect of PDGF, IGF-1 and PRP on the implant osseointegration. An histological and immunohistochemical study in rabbits. Ann Stomatol 2014;5:66–8.
[34] Song D, Shujaat S, Huang Y, Van Dessel J, Politis C, Lambrichts I, Jacobs R. Effect of platelet-rich and platelet-poor plasma on 3D bone-to-implant contact: a preclinical micro-CT study. Int J Implant Dent 2021;7:11.
[35] Oncu E, Erbeyoglu AA. Enhancement of immediate implant stability and recovery using platelet-rich fibrin. Int J Periodontics Restor Dent 2019;39:e58–63.
[36] Taschieri S, Lolato A, Ofer M, Testori T, Francetti L, Del Fabbro M. Immediate post-extraction implants with or without pure platelet-rich plasma: a 5-year follow-up study. Oral Maxillofac Surg 2017;21:147–57.
[37] Marx RE, Garg AK. Dental and craniofacial applications of platelet-rich plasma. Chicago, USA: Quintessence Pub. Co.; 2005. p. 1–154.
[38] He L, Lin Y, Hu X, Zhang Y, Wu H. A comparative study of platelet-rich fibrin (PRF) and platelet-rich plasma (PRP) on the effect of proliferation and differentiation of rat osteoblasts in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;108:707–13.
[39] Graziani F, Ivanovski S, Cei S, Ducci F, Tonetti M, Gabriele M. The in vitro effect of different PRP concentrations on osteoblasts and fibroblasts. Clin Oral Implants Res 2006;17:212–9.
[40] Liu Y, Kalen A, Risto O, Wahlstrom O. Fibroblast proliferation due to exposure to a platelet concentrate in vitro is pH dependent. Wound Repair Regen 2002;10:336–40.