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# Injectable platelet rich fibrin facilitates hair follicle regeneration by promoting human dermal papilla cell proliferation, migration, and trichogenic inductivity

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ABSTRACT

Hair follicle regeneration has been successful in mice but failed in human being for years. Dermal papilla cells, a specialized mesenchymal stem cell derived from dermal papilla within hair follicles, is considered the key cells for hair follicle regeneration function as both regeneration initiator and regulator. Injectable platelet rich fibrin (i-PRF), a novel biomaterial rich in a variety of growth factors and three-dimensional scaffolds, has shown promising effects on tissue regeneration. In this study, we aimed to evaluate the application of i-PRF in human hair follicle regeneration by examining the biological effects of i-PRF on human dermal papilla cells (hDPCs). Biomaterial compatibility, cell viability, proliferation, migration, alkaline phosphatase activity and trichogenic inductivity were assessed after exposing hDPCs to different concentrations of i-PRF extracts. In addition, we investigated the ultrastructure of i-PRF with all cell components filtered. The results revealed that i-PRF possessing excellent biocompatibility and could significantly promote hDPCs proliferation, migration, and trichogenic inductivity. Furthermore, the concentration of i-PRF is able to remarkably influence hDPCs behavior in a dose-dependent pattern. Different concentrations exhibited differential effects on hDPCs behavior. In general, lower concentration promotes cell proliferation better than higher concentration, while higher concentration promotes cell function better reversely. Best concentration for hDPCs in vitro expending is 1% concentration. 20% concentration is optimal for hair follicle regeneration. In summary, our findings concluded that i-PRF facilitates hair follicle regeneration by promoting human dermal papilla cell proliferation, migration, and trichogenic inductivity.

## 1. Introduction

As a feature of mammalians, hair plays a great role in body warmth, protection, touch sense, and social communication. The turnover of hair follicles (HF) occurs in cycles with alternating stages of rapid growth and hair shaft formation (anagen), apoptosis-driven regression (catagen) and relative HF quiescence (telogen) [1]. Throughout the entire cycle, the cross-talk between two main compartments, epithelial and mesenchymal, is the key to a proper hair growth rhythm [2]. In general, the reciprocal and complicated cross-talk is induced and regulated by dermal papilla cells (DPCs) which is a specialized fibroblast derived from dermal mesenchyme acting as a reservoir of multipotent stem cells, nutrients and growth factors. Previous studies have successfully induced hair follicle regeneration by transplanted DPCs in mice, while the same experiments failed in human being [3–7]. Promoting human dermal papilla cells (hDPCs) behavior especially trichogenic inductivity have become the intense focus of hair biology [8]. Additionally, dermal papilla cells are capable of differentiating into other cell types such as

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adipocyte, osteoblasts and chondrocytes making these cells a more attractive cell seed for tissue regeneration [9].

Capable of substantially releasing supraphysiological doses of growth factors, platelet concentrates have been utilized as an autologous regenerative tool in dentistry for over five decades [10,11]. Since then, diverse production protocols were introduced resulted in various products being widely used beyond dentistry. Among them, platelet rich plasma (PRP) was considered the first generation introduced by Kingsley et al., in 1954 [12]. However, the use of bovine thrombin and various other anticoagulants, known suppressors of tissue regeneration, has raised great concerns [13]. For these reasons, platelet rich fibrin (PRF), the second generation of platelet concentrates produced without any anticoagulants, was developed guaranteeing a prolonged release of growth factors and cytokines and a three-dimensional scaffold structure for cell culture [14]. But the solid form of PRF limits its application. In 2014, an injectable platelet rich fibrin (i-PRF) was developed by modifying spin centrifugation forces based on low speed centrifugation concept (LSCC) [15,16]. This purely autologous platelet concentrate maintains a liquid viscosity for approximately 15 min before forming a fibrin clot giving it a widespread utility [17]. Having the advantages of the former two generations, i-PRF was considered the third generation of platelet concentrates possessing a higher and longer release of a variety of growth factors, cytokines and a delicate three-dimensional scaffold structure [18].

Although platelet concentrates was well-known by dentistry and used in stomatology mostly, recent evidences have shown a great application prospect for i-PRF in other medical fields [5,19–22]. Currently, platelet concentrates have been regarded as a promising strategy for hair follicle regeneration due to the special ability of growth factor release and the three-dimensional scaffold structure [23]. Recent studies have shown the concentration of platelet concentrates demonstrated a strong influence on cell behavior [18]. Higher concentration was considered detrimental while the optimal concentration within the range of 1%–30% remains controversial [18,24–26]. This work was undertaken to investigate the effects of i-PRF on human dermal papilla cells (hDPCs) and define the most optimal concentration for hair follicle regeneration. To the best of our knowledge, this is the first study investigating the effects of i-PRF on human dermal papilla cells.

## 2. Materials and methods

### 2.1. Ethics

All experimental procedures in this study were performed in accordance with the guidelines and regulations established by the medical ethics committee of Northern Jiangsu People's Hospital.

### 2.2. Preparation of i-PRF with different concentrations

Peripheral venous blood samples were collected from 8 healthy volunteers (age range 22–28, 4 males and 4 females) upon signing an informed consent form. Smokers or volunteers with any abnormality of biochemical tests results were excluded. For i-PRF preparation, 10 ml of whole blood without anti-coagulant was centrifuged at 60 g RCF for 3 min at room temperature in a plastic tube, 60 g RCF is equivalent to 700 rpm for devices with a 110 mm radius, and 1 ml of i-PRF was collected from the upper layer sequentially. The collected i-PRF was transferred to a 6-well plate (Corning) to form a complete clot for 1.5 h before incubation with 5 ml DMEM (Gibco) without any supplements at 37 °C in a 5% CO2 incubator for 72 h. After incubation, the medium was aspirated and considered 100% i-PRF conditioned medium. The collected 100% i-PRF conditioned medium was then carefully diluted with standard culture medium (DMEM supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum) to create 5 different concentrations of i-PRF conditioned medium: 1% (v/v); 5% (v/v); 10% (v/v); 20% (v/v); 30% (v/v). All conditioned mediums were stored at −80 °C until use for the subsequent characterization and biological assays.

### 2.3. Isolation & culture of human dermal papilla cells ( hDPCs )

Human intact scalp skin was obtained during surgical removal of benign skin tumors with informed consent. Dermal papilla cells were isolated and expanded as previously described [27]. Briefly, dermal papillae were obtained by microdissection of the hair follicle transected from the human scalp skin and transferred to 35 mm culture dishes (Corning) and cultured in DMEM (Gibco) supplemented with 1% (v/v) penicillin-streptomycin and 20% (v/v) fetal bovine serum (Gibco) in a humidified 5% CO2 incubator at 37 °C. After 10 days without any movement and medium changes, when dermal papilla cells were successfully migrated out, culture medium was changed every 3 days until the outgrowth had reached 80% confluence. Human dermal papilla cells (hDPCs) were then harvested by incubation with 0.25% (w/v) trypsin/EDTA (Gibco) and transferred to a new 25 $cm^2$ culture flask (Corning) with a split ratio of 1:2. Thereafter, dermal papilla cells (hDPCs) were maintained in DMEM (Gibco) supplemented with 1% (v/v) penicillin-streptomycin and 10% (v/v) fetal bovine serum (Gibco). hDPCs at passage 2 (P2) were used in the following experiments. A total of three primary cell cultures were used for the following experiments for three independent replicates.

### 2.4. Scanning electron microscopy (SEM)

In order to investigate the details of the three-dimensional scaffold of i-PRF, SEM examination was performed with all cell elements filtered by Millex® filters with a pore size of 0.22 μm (Merck, Sigma-Aldrich, Germany). Briefly, the i-PRF samples immediately after completing their clot formation were fixed in 2% glutaraldehyde in Dulbecco's phosphate buffered saline (DPBS) buffer with a pH of 7.4 overnight. Next, samples were dried in the desiccator, then sputtered with 20 nm gold. Ultrastructure properties of i-PRF scaffold was assessed by SEM then.

### 2.5. Characterization of the cultivated human dermal papilla cells (hDPCs)

The P2 and P8 hDPCs cultured in the 25 $cm^2$ flasks (Corning) were observed under an inverted microscope (Zeiss, Germany) and photographed. Alkaline phosphate stain was performed as following part "Alkaline phosphatase activity" introduced. For immunofluorescence stain, the P2 hDPCs were plated on glass slides (YA0352, Solarbio, China) at a density of $6.0 \times 10^4$ cells/well and fixed with 4% paraformaldehyde (BL539A, Biosharp, China) for 15 min. After washing by PBS, the cells were permeabilized with 1% Triton X-100 (Beyotime, China) for 10 min, blocked with 5% BSA for 10 min at room temperature, and incubated with mouse monoclonal anti-α-SMA antibody (ab7817, Abcam, UK), mouse monoclonal anti-Versican antibody (sc-47769, Santa Cruz, USA), and mouse monoclonal anti-Cytokeratin 8 antibody (sc-8082, Santa Cruz, USA) overnight at 4 °C. Thereafter, the cells were incubated with goat anti-mouse IgG secondary antibody (ab150133, Alexa Fluor 488, Abcam, UK) and goat anti-mouse IgG secondary antibody (ab150116, Alexa Fluor 594, Abcam, UK) respectively for 2 h at room temperature and washed by PBS before counterstaining with DAPI (Beyotime, China) for cell nuclei. Finally, the fluorescence signals were observed under an inverted fluorescence microscope. (Zeiss, Germany).

### 2.6. Cell viability assay

Cells were plated onto 6-well plates at a density of $9.0 \times 10^4$ cells/well and incubated for 24 h in a humidified 5% CO2 incubator at 37 °C. Thereafter, the medium was aspirated and changed into i-PRF conditioned mediums with different concentrations. After 24 h, a live-dead

staining assay was performed to evaluate the cells cultured with different concentrations of i-PRF conditioned mediums using a Calcein/PI Cell Viability Assay Kit (C2015S, Beyotome, Shanghai, China). Fluorescent images were quantified with an inverted fluorescent microscope (Zeiss, Germany). Finally, cells were expressed as percentages of live cells (green) versus dead cells (red) using the following formula. Cell viability (%) = number of live cells/(number of live cells + number of dead cells) x 100.

### 2.7. Cell proliferation assay

Cells were seeded in 96-well plates at a density of 1000 cells/well. After cell adherence, the medium was replaced by different concentrations of i-PRF conditioned mediums. Cells in the control group were treated with a standard culture medium (DMEM supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum). The medium was changed every 3 days, for a total culture period of 7 days. And a cell counting kit-8 (CCK8, C0038, Beyotime, China) was used following the manufacturer's protocol. The optical density (OD) at 450 nm was measured and recorded at different time points (1 day, 3 days, 5 days, and 7 days of culture). Three independent biological replicates were assessed. To detect the proliferative ability of hDPCs, 10 μM EdU solution was added to the medium for 18 h. Using the BeyoClick EdU Cell Proliferation Kit with Alexa Flouor 488 (Beyotime, Shanghai, China), Hoechst 33,342 was used for nuclear staining according to the manufacturer's instructions. Cells stained with both green and blue were considered EdU-positive cells.

### 2.8. Cell migration assay

Boyden chamber assay was used to evaluate the cell migration ability. After starvation, hDPCs at a density of $6 \times 10^4$ cells/well were seeded into the upper chamber of the polyethylene terephthalate (PET) cell culture inserts with an 8 μm pore size (Corning, New York, NY, USA). The lower chamber of the well was filled with i-PRF conditioned mediums with different concentrations. Cells were allowed to migrate for 24 h before being fixed with 4% paraformaldehyde (BL539A, Biosharp, China) for 15 min. Cells were stained with Crystal Violet Staining Solution (C0121, Beyotime, Shanghai, China) for 10 min, and the non-migrated cells on the upper chamber were removed with a cotton swab. Migrated cells were observed and photographed by an inverted microscope (Zeiss, Germany). The numbers of cells migrated were counted with Image J software.

### 2.9. Alkaline phosphatase activity

To determine the alkaline phosphatase activity of hDPCs cultured with different concentrations of i-PRF conditioned mediums. A BCIP/NBT Alkaline Phosphatase Color Development Kit (C3206, Beyotime, Shanghai, China) was used according to the manufacturer's instructions. Briefly, cells were fixed with 4% paraformaldehyde (BL539A, Biosharp, China) for 15 min at temperature. After washing with PBS, cells were stained incubated with 4-Nitro blue tetraxolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphospate (BCIP) diluted in NTMT buffer for 20 h and observed and photographed sequentially.

### 2.10. Real-time PCR

For real-time PCR experiments, total RNAs were isolated from hDPCs cultured with different concentrations of i-PRF conditioned medium after 14 days using an RNApure Tissue Kit (DNase I) (CW0560S, CWBIO, China) following the manufacturer's instructions. A total of 1 μg RNA solution was immediately reverse transcribed to cDNA using a HiScript® III RT SuperMix for qPCR kit (R323-01, Vazyme, China) and the final volume is 100 μl. The sequences of primers were listed in Table 1, including bone morphogenetic protein 4 (BMP4); hes related family bHLH transcription factor with YRPW motif 1 (HEY1); bone morphogenetic protein 2 (BMP2); fibroblast growth factor 10 (FGF10); fibroblast growth factor 7 (FGF7); serpin family E member 1 (SERPINE1); endothelin 1 (EDN1); insulin like growth factor 1 (IGF1); versican (VCAN); alpha smooth muscle actin (a-SMA); Wnt family member 5A (WNT5A); lymphoid enhancer binding factor 1 (LEF1); noggin (NOG); alkaline phosphatase (ALP); and GAPDH genes of human. Real-time RT-PCR was performed using 20 ml final reaction volume of Taq Pro Universal SYBR qPCR Master Mix kit (Q712-02, Vazyme, China) following the manufacturer's protocols.

### 2.11. Statistical analysis

All experimental data were validated by three replicates and expressed as mean ± standard deviation (SD) using one-way ANOVA test and two-way ANOVA test dependently by Graphpad Prism 9.0 software. P values less than or equal to 0.05 were considered statistically significant.

## 3. Results

### 3.1. Characterization of the i-PRF scaffold

The examination of the i-PRF scaffold by SEM showed a three-dimensional fibrin network architecture (see Fig. 1).Main fibrin fiber bundles were arranged in one direction with a lot of fibrin fiber branches spreading out, connecting and crossing each other. Within the complex fibrin network, pores sizing from 10 μm to 30 μm were found which channeled into each other and build a connected scaffold world for cells. Coarse surfaces of fibrin fibers were observed within the delicate

**Table 1**
Real-time PCR primer sequences.

| Gene | Forward primer (5′ to 3′) | Reverse primer (5′ to 3′) |
|---|---|---|
| HEY1 | GGCAGGAGGGAAAGGTTACTT | GAAGCGTAGTTGTTGAGATGCG |
| BMP4 | CGGACTACATGCGGGATCTT | CACCTCGTTCTCAGGGATGC |
| FGF10 | CTTGGTGTCTTCCGTCCCTGT | GCAACAACTCCGATTTCTACTGATG |
| SERPINE1 | CCCCACTTCTTCAGGCTGTT | GCCGTTGAAGTAGAGGGCAT |
| EDN1 | CTGCCACCTGGACATCATTTG | GCACATTGGCATCTATTTTCACG |
| IGF1 | TGCCTTGGTCTCCTTGTCCT | GTCACTTCCTCCATGCGGTAA |
| GAPDH | GGAAGCTTGTCATCAATGGAAATC | TGATGACCCTTTTGGCTCCC |
| α-SMA | CAATGTCCTATCAGGGGGCAC | CGGCTTCATCGTATTCCTGTT |
| VCAN | TATGGAGATAAGATGGGAAAGGC | CCGTAATCGCACTGGTCAAA |
| WNT5A | AATTCTTGGTGGTCGCTAGGTAT | ACCTTCGATGTCGGAATTGATAC |
| BMP2 | GCAGCTTCCACCATGAAGAAT | GGAATTTCGAGTTGGCTGTTG |
| FGF7 | GGACAGTGGCAGTTGGAATTG | CCATAGGAAGAAAGTGGGCTGT |
| LEF1 | GCGAATGTCGTTGCTGAGTGTA | GCTGTCTTTCTTTCCGTGCTAA |
| NOG | CGGAGGAAGTTACAGATGTGGCT | ACTTGCACTCGGAAATGATGG |
| ALP | AAAGTCCTTCAAAGCTGGAGTCTG | ACATCTCTTCTTCCCTGGACACTG |

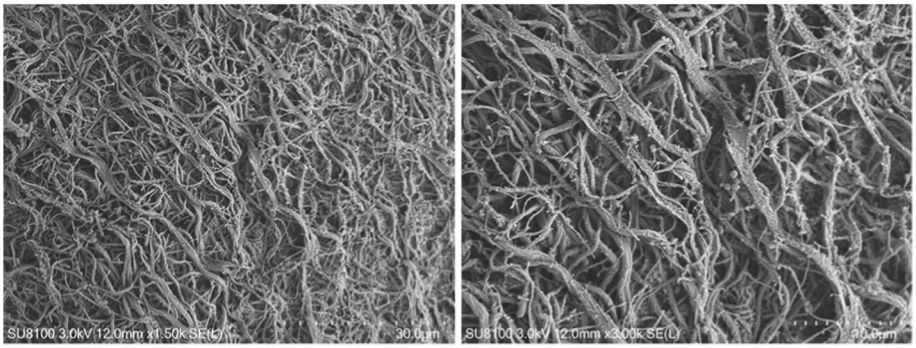

**Fig. 1.** Scanning electron micrograph of decellularized i-PRF scaffold. Main fibrin fiber bundles arranged in one direction with lots of fibrin fiber branches spreading, connecting and crossing each other. Pores sizing from 10 mm to 30 mm channeled into each other. Scale bars = 30 μm (A) Scale bars = 10 μm (B).

scaffold for cell adhesion. These observations confirmed i-PRF as an optimal biological scaffold.

### 3.2. Characterization of the cultivated hDPCs

Fig. 2A–C presents the characteristic cell morphology of cultivated human dermal papilla cells. At passage 2, human dermal papilla cells grow in a sunflower-like radial pattern and aggregated to form papilla-like structures (Fig. 2A and 2B). hDPCs at passage 8 failed to form papilla-like structures and grows in an overlying pattern (Fig. 2C). Alkaline phosphatase activity was demonstrated by results of alkaline phosphatase (ALP) staining (Fig. 2 D). Immunofluorescent imaging data was presented as α-SMA (Fig. 2E–G), Versican (Fig. 2H–J), Cytokeratin 8 (Fig. 2K-M) expression after culture.

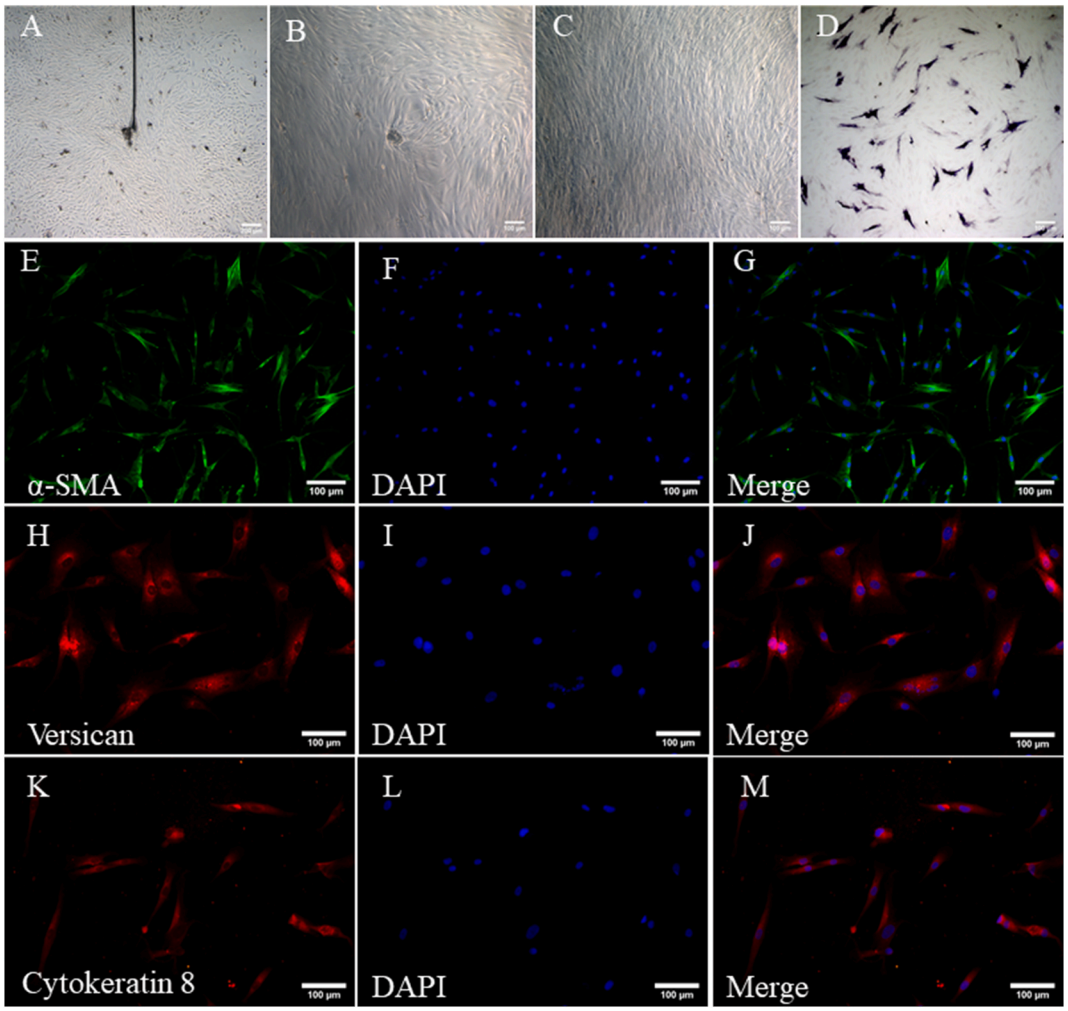


**Fig. 2.** The characteristics of cultured hDPCs. **A** Cultured primary hDPCs were arranged in a radial pattern and showed a typical morphology of fibroblast cells with a spindle-like shape. **B** Cultured hDPCs aggregated to form a dermal papilla-like structure at passage two. C Cultured hDPCs lost their trichogenic inductivity to form dermal papilla-like structures and grew in a whirlpool with cells overlapping each other at passage eight. **D** Alkaline phosphatase activity (violet) results of hDPCs at passage two. **E, H, K** Immunofluorescent imaging data was presented as α-SMA (green), Versican (red), and Cytokeratin 8 (red) expression after culture. **F** Nuclei were stained with DAPI (blue) and **G** pseudo colored merged image. Scale bars = 100 μm.

### 3.3. Effects on hDPCs viability

At first, the biocompatibility of different concentrations of i-PRF was evaluated with live/dead assay. It was found that all concentrations of i-PRF demonstrated excellent cell viability and biocompatibility, as demonstrated by the high percentage of living cells (>95%, green cells, Fig. 3B) and few observable dead cells (red cells, Fig. 3B) with no significant difference among groups (Fig. 3A). It may be concluded therefore that all concentrations of i-PRF displayed excellent cell viability and biocompatibility with respect to cell survival.

### 3.4. Proliferative effects on hDPCs

As presented by Fig. 4A and C, EdU positive cells were significantly decreased as the concentration of i-PRF conditioned medium increased, showing a dose-dependent effect on hDPCs with lower concentration promotes proliferation better. Cell proliferation effects of different concentrations were tested by CCK-8 assay at different time points (Day1, Day3, Day5, Day7). Lower concentrations (1%, 5%) has shown great promoting effect throughout the 7 days. Medium concentration (10%) was moderate from day 1 to day 7. While toxic effects of higher concentrations (20%, 30%) were observed from day 3 (Fig. 4B). These results may indicate that a lower concentration of i-PRF was more suitable for cell proliferation.

### 3.5. Trans-well migration

A *trans*-well migration assay was used to examine the effects of different concentrations of i-PRF conditioned medium on hDPCs migration. As shown in Fig. 5, hDPCs migration was significantly stimulated by higher concentrations (10%, 20%, 30%) with a dose-dependent effect. No significance was observed when hDPCs were treated with lower concentrations (1%, 5%) compared with control group. Therefore, it may be concluded that i-PRF promotes hDPCs migration with a dose-dependent effect, higher concentration shows a better promoting effect.

### 3.6. Alkaline phosphatase activity of hDPCs

Since alkaline phosphatase activity is a key indicator of hDPCs trichogenic inductivity, an ALP stain was performed to evaluate the effect of different concentrations of i-PRF on hDPCs. Presented by Fig. 6, the staining was deeper as concentration went higher suggesting ALP activity was also higher when hDPCs were treated with a higher i-PRF conditioned medium. In general, higher concentrations (10%, 20%, 30%) exhibited much deeper staining, suggesting a higher alkaline phosphatase activity.

### 3.7. Gene expression of cultivated hDPCs

Lastly, to investigate the effects of different concentrations of i-PRF on hDPCs trichogenic inductivity, hDPCs were treated with different concentration subgroups for 7 days and 14 days, mRNA levels of genes related were evaluated by real-time PCR sequentially (Fig. 7). At 14 days, higher concentrations (10%, 20%, 30%) exhibited significant upregulating effects on gene expressions of Notch signal pathway (HEY1), BMP signal pathway (BMP2, BMP4, Noggin), FGF signal pathway (FGF7, FGF10), WNT signal pathway (LEF1, WNT5A), growth factors (IGF-1, Endothelin 1), ALP activity (ALP) and hDPCs signature genes (a-SMA, VCAN, Nexin 1), while lower concentrations (1%, 5%) barely showed upregulation effects on WNT pathway (WNT5A) and inhibiting effects on BMP pathway (BMP4, 1%). Within each aspect, the optimal concentration varied among concentration groups. In general, the highest expression was observed with 20% concentration followed by a decrease with 30% concentration for Notch signal pathway (HEY1), BMP signal pathway (BMP2, BMP4), WNT signal pathway (LEF1, WNT5A), growth factors (IGF-1, Endothelin 1), ALP activity (ALP) and hDPCs signature genes (a-SMA, VCAN), however, it was worth mentioning that 30% concentration showed a special powerful upregulating effect on Nexin 1 gene expression. And medium concentration (10%) subgroup showed a unique promoting effect on FGF signal pathway (FGF7, FGF10) in addition. At last, lower concentrations (1%, 5%) failed to show significant results in summary. The same trend was observed but less powerful at 7 days (data not shown). (Fig. 7).

## 4. Discussion

As a promising regenerative tool, i-PRF is a purely autologous three-dimensional living biomaterial rich in growth factors that can modulate regenerative processes, possessing improved biological properties compared to last two platelet concentrates generations [13]. Previous studies have shown a promising application aspect of i-PRF in hair restoration while the exact effects remain unknown. In addition, studies focused on the influence of concentration of i-PRF on different cells have been reported with controversial results. At first, it is widely acknowledged that toxic effect will overwhelm when the growth factors concentration is too high, proved by the concentration of fetal bovine serum within the culture medium which is carefully set around 10%–20%.

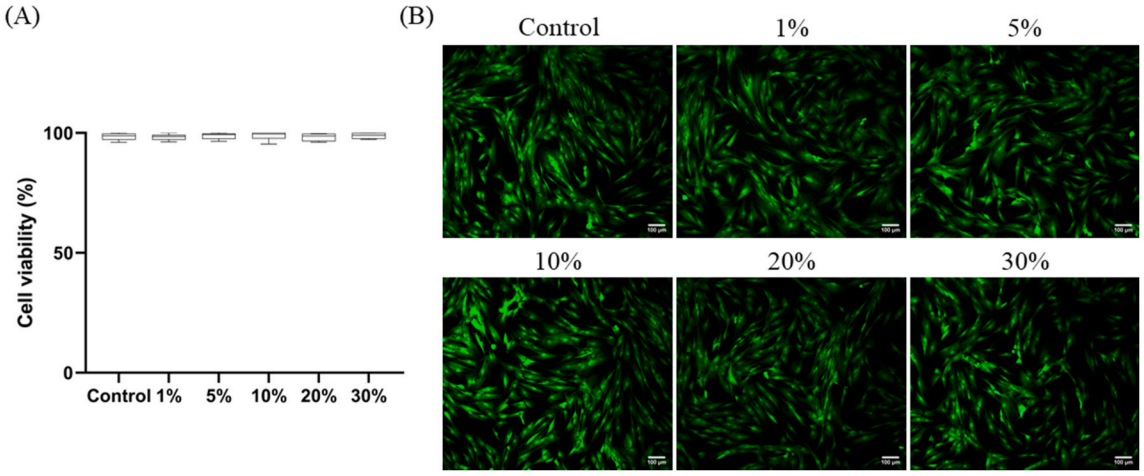


**Fig. 3.** Live/dead assay of hDPCs treated with i-PRF conditioned mediums with different concentrations at 24 h. (A) Cell viability was quantified as the percentage of living cells in each group. No significant differences were observed among the three groups. (B) Merged fluorescence images show the results of the live/dead staining with viable cells appear green and dead cells appear red. Scale bars = 100 μm.

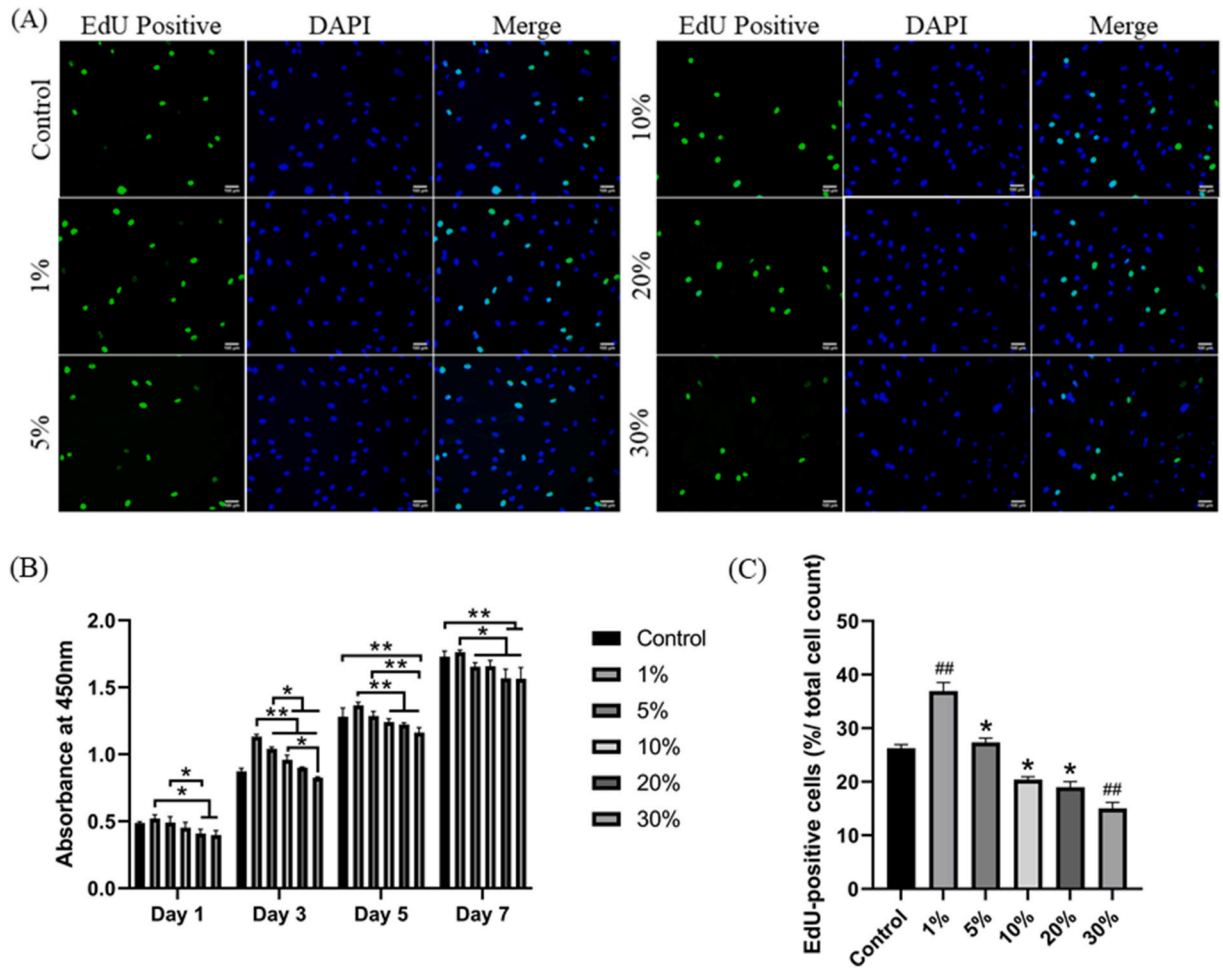


**Fig. 4.** The proliferative effects of different concentrations of i-PRF conditioned mediums on hDPCs. (A) The fluorescence images of EdU incorporation assay showed that the proliferative cells (green, EdU positive) were less frequently detected as the concentration of i-PRF conditioned mediums increased. (B) CCK-8 assay showed lower concentration promotes hDPCs proliferation better then higher concentration during 7 days culture. $^{*}p < 0.05$; $^{**}p < 0.01$ (C) Comparative analysis of the ratios of EdU-positive cells among all the groups confirmed the proliferative effects decreased as the concentration of i-PRF conditioned mediums increased. $^{*}p < 0.05$ compared with the control group; $^{\#\#}p < 0.01$ compared with all other groups. Scale bars = 100 μm.

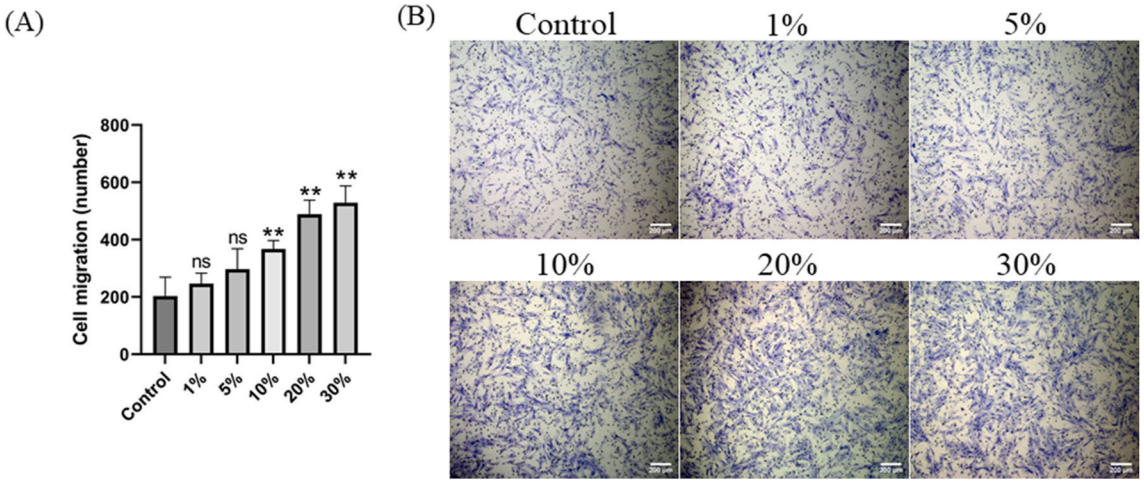


**Fig. 5.** *Trans*-well migration of hDPCs cultured with different concentrations of i-PRF conditioned mediums. (A) Comparative analysis of the cell migrated showed higher concentrations significantly promoted cell migration. $^{**}p < 0.01$; ns: no significance. (B) Migrated hDPCs were stained violet by Crystal Violet Staining Solution, migrated cells increased as the concentration of i-PRF conditioned mediums increased. Scale bars = 200 μm.

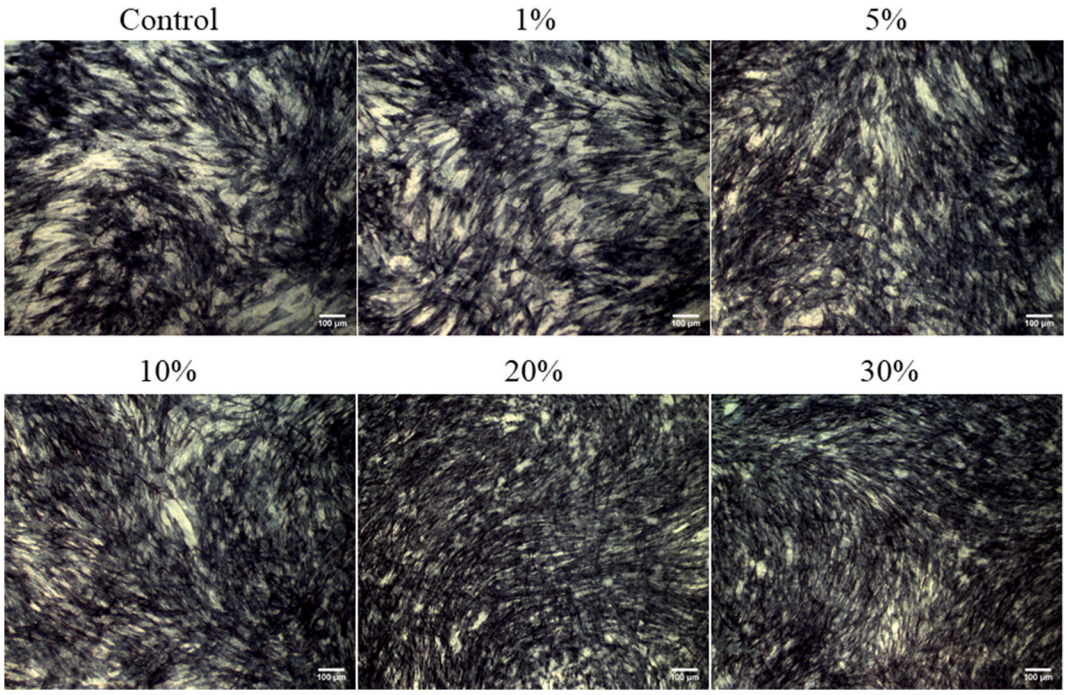


**Fig. 6.** ALP activity of hDPCs treated with different concentrations of i-PRF conditioned medium. ALP stain was deeper as the concentration went higher. Concentrations (5%, 10%, 20%, 30%) exhibit deeper stain compared with control group. Scale bars = 100 μm.

Similar results were observed with platelet concentrates, Fernández--Medina et al. [18] systematically investigated 5 concentrations (20%, 40%, 60%, 80%, 100%) of different platelet concentrates and results confirmed a concentration over than 40% was detrimental. Researchers believed that the optimal concentration was within the range of 1%–30%, while studies focused on this range reported totally opposite effect. Higher concentration over than 10% was considered beneficial in some studies [25,28,29], while the other studies suggested the opposite [13,24,30–32]. However, all of these studies mentioned about their limits in concentration group setting, either lack of higher concentration groups (≥20%) or lower concentration groups (≤5%). In the present study, we carefully set a full-scale concentrations group (1%, 5%, 10%, 20%, 30%). And the effects of different concentrations of i-PRF on hDPCs viability, proliferation, migration, ALP activity and trichogenic inductivity related genes expression were investigated.

Previously, PRP and PRF, the first and second generations of platelet concentrates, have been utilized in tissue regeneration of dentistry and showed satisfactory biocompatibility. Our study found similar results, all concentration groups demonstrated great cell viability (>95%) after 24 h of culture and no significant difference was observed. i-PRF was proved an ideal biocompatible material for human dermal papilla cells. For cell proliferation, EdU proliferation assay and CCK8 proliferation assay were performed where a dose-dependent effect of i-PRF concentration was found. Lower concentration seems to promote cell proliferation better. Results showed 1% concentration significantly stimulated hDPCs proliferation compared with other groups. As a key center for hair cycle modulation and an important cell seed for tissue regeneration, human dermal papilla cells are quite precious and usually obtained in a limited amount. 1% concentration of i-PRF displayed promising biocompatibility and cell proliferation promoting ability and was considered the optimal concentration for hDPCs in vitro cell expansion.

It has been reported that trichogenic inductivity enhanced when cultured hDPCs form dermal-papilla like structures in vitro [5]. In this study, the migration of cells was investigated using a *trans*-well assay with hDPCs treated by different concentrations of i-PRF. Results revealed a dose-dependent effect that higher concentration of i-PRF significantly stimulates cell migration with 30% as the optimal concentration. For ALP activity, similar dose-dependent effect was observed in ALP stain, higher concentration of i-PRF exhibited deeper staining color indicating a stronger ALP activity. Furthermore, intrinsic characteristics of hDPCs especially trichogenic inductivity influence was investigated with real-time PCR. It is well acknowledged that intrinsic characteristics of hDPCs altered during traditional culture and the trichogenicity of hDPCs lost as well. Prior studies have identified lots of genes related to trichogenic inductivity of hDPCs [5,33,34]. There are four signal pathways involved including Notch signal pathway, FGF signal pathway, BMP signal pathway, and WNT signal pathway. For each signal pathway, we carefully selected representative genes for testing based on prior publications. Apart from signal pathways, these genes also involved growth factors producing and alkaline phosphatase activity which we also carefully selected representative genes. Moreover, there are several signature genes of hDPCs do not belong to any of the categories above, we subsumed these genes under the signature genes. The results proved injectable platelet rich fibrin (i-PRF) an ideal biomaterial for hair follicle regeneration which promptly enhanced hDPCs trichogenic inductivity. Surprisingly, differential effects were observed for different concentrations of i-PRF on hDPCs both in 7 days (data not shown) and 14 days. In general, higher concentration over than 10% stimulated gene expression significantly, while concentrations lower than 10% showed no significance. For signal pathways, the 20% concentration exhibited the greatest effects on WNT signal pathway, Notch signal pathway and part of BMP signal pathways. While the 30% concentration showed a distinctive promoting effect for Noggin gene expression compared with other selected genes in BMP signal pathway. What's more, the 10% concentration displayed the greatest effects on FGF signal pathways in comparison with other signal pathways. We assume a slightly higher concentration than 20% might be more suitable for BMP signal pathway stimulation while a lower concentration might be better for FGF signal pathway stimulation. As for growth factor producing and alkaline phosphatase activity, the 20% concentration exhibited the greatest effects once again. However, when referring to the signature genes categories, the effects of different concentrations was different, the 20% concentration displayed the greater stimulating effects on most signature genes expression while the 30% concentration

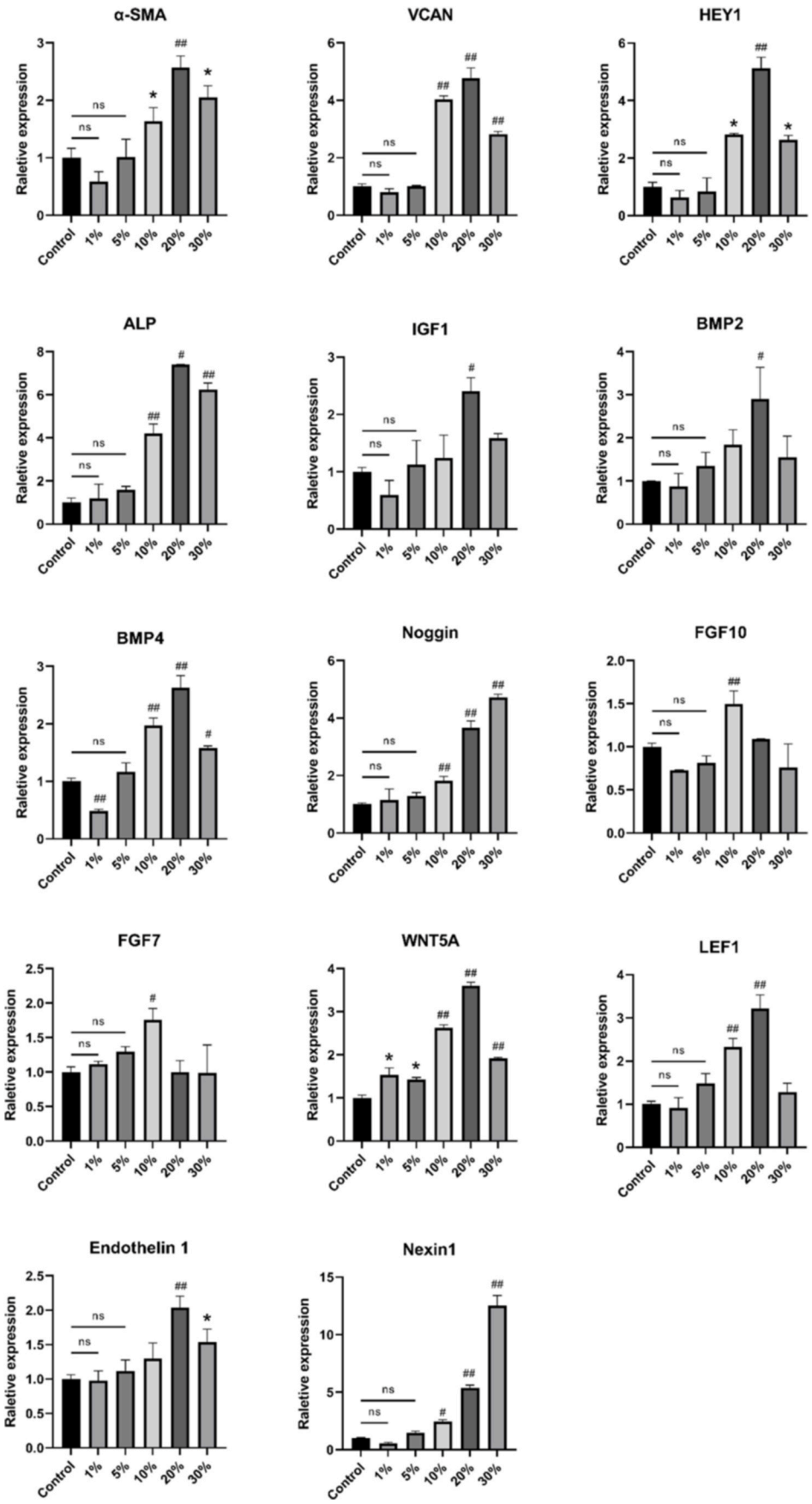


**Fig. 7.** Gene expressions of hDPCs treated with different concentrations of i-PRF conditioned medium. In general, higher concentrations (10%, 20%,30%) significantly upregulated expression of genes related to Notch signal pathway (HEY1), BMP signal pathway (BMP2, BMP4, Noggin), FGF signal pathway (FGF7, FGF10), WNT signal pathway (LEF1, WNT5A), growth factors (IGF-1, Endothelin 1), ALP activity (ALP) and hDPCs signature genes (α-SMA, VCAN, Nexin 1), while lower concentrations (1%,5%) barely showed significant effects. In addition, different optimal concentrations were observed within different aspects. $^{*}p < 0.05$ compared with control group. $^{\#}p < 0.05$ compared with all other groups. $^{\#\#}p < 0.01$ compared with all other groups. ns: no significance.

stimulated the Nexin 1 gene expression powerfully. In a conclusion, although with some variations, still we considered the 20% concentration as the optimal concentration for hDPCs trichogenic inductivity in general.

In summary, the present study concludes that i-PRF, the third generation of platelet concentrates, is an ideal biocompatible material for hair restoration and the concentration of i-PRF is able to remarkably influence hDPCs behavior in a dose-dependent pattern. Lower concentration promotes cell proliferation better, while higher concentration promotes cell function better. The optimal concentration recommended for cell expansion is 1% and 20% concentration is recommended as the optimal concentration for cell function. However, as i-PRF is invented for clinical usage, further researches of animals and clinical studies across many fields of medicine are required to full confirm the potential of i-PRF as a new platelet concentrates.

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## Institutional Review Board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Northern Jiangsu People's Hospital (protocol code 2020ky-050; 2020/08/17).

## Informed consent statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

## Author statement

**Kongye Lu:** Conceptualization, Methodology, Software, Writing – original draft. **Qiwen Han:** Visualization, Investigation, Software. **Zekun Ma:** Data curation, Investigation. **Qingqing Yan:** Investigation, Software, Validation. **Yunlong Pei:** Investigation, Data curation. **Pengzhi Shi:** Investigation, Data curation. **Jin Zhang:** Investigation, Resources, Data curation. **Kunjie Rong:** Resources. **Kun Ma:** Resources. **Pingsong Li:** Conceptualization, Data curation, Writing – review & editing, Project administration. **Tuanjie Hou:** Conceptualization, Writing – review & editing, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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