Int J Clin Exp Pathol 2017;10(12):11703-11709
www.ijcep.com /ISSN:1936-2625/IJCEP0035241

Original Article

Platelet-rich plasma promotes cell viability of human hair dermal papilla cells (HHDPCs) in vitro

Yuyan Wang, Liang Tang, Fei Zhu, Ming Jia

Department of Plastic Surgery, Hangzhou First People's Hospital, Nanjing Medical University, Hangzhou, Zhejiang 310006, P.R China

Received July 7, 2016; Accepted September 21, 2016; Epub December 1, 2017; Published December 15, 2017

Abstract: Purpose: This study aimed to clarify the effect of platelet-rich plasma (PRP) on human hair dermal papilla cell (HHDPC) viability and expression levels of alkaline phosphatase (ALP) and versican. Methods: Eight healthy volunteers were enrolled, and different concentrations of PRP and platelet-poor plasma (PPP) were preparedby 2-step centrifugation method. HHDPCs were thencultivated. To identify the best incubating time of HHDPCs and plasmaconcentration, cell viability was investigated by MTT assays. In PRP- or PPP-treated HHDPCs, the mRNA levels of versican and ALP were checked by real-time quantitative PCR (qRT-PCR). Results: Both PRP (5% and 10%) and PPP (5% and 10%) could significantly promote cell viability of the HHDPCs (p4) in 72 h group, while the effect of 10% PRP on HHDPC viability was less than that of 5% PRP. qRT-PCR showed that PRP could significantly increase mRNA levels of ALP and versican in HHDPCs (p4) when compared with the control group. Meanwhile,Western blot indicated an obviously increased protein level of ALP and versican in PRP-treated HHDPCs (p4). Conclusion: PRP could promote cell viability of HHDPCs. Besides, 5% PRP could significantly increase ALP and versican expression in both mRNA and protein levels in HHDPCs (p4).

Keywords: Platelet-rich plasma, platelet-poor plasma, human hair dermal papilla cells, MTT assay

Introduction

Hair follicleregeneration, which is composed by anagen, catagen, telogen and exogen [1], is of great importance in treating dermal wound and alopecia [2, 3]. Alopecia (also known as hair loss) has many risk factors, such asnutritional deficiencies, traumatic damage and fungal infection [4]. Besides, dermal wound can result in significant disability or even lead to death [5, 6]. Along with increased mechanical trauma, burns, skin disease and ageing, dermal wound healing is becoming more and more urgent [5, 7].

Compared with whole blood, platelet-rich plasma (PRP) is blood plasma that enriched with platelets, and platelet-poor plasma (PPP) is blood plasma with low-level platelets. Alpha granules in platelets contain plenty of growth factors that can promote cell proliferation and differentiation, thus PRP has more growth factors than PPP [8]. Researchers have found that PRP can stimulate mesenchymal stem cell (MSC) proliferation and induce MSC differentiating to chondrocyte in vitro [9-11]. Previous studies report that PRP has a considerable effect on hair induction and hair follicle regeneration [12, 13]. In addition, dermal papilla cells (DPCs, a kind of dermal cells in hair follicle) can induce hair growth and hairfollicleregeneration [14]. However, the effect of PRP on human hair dermal papilla cells (HHDPCs) viability and hair follicle regeneration were stillunclear.

In this study, different concentrations of PRP and PPP were prepared, and HHDPCs were cultivated. Afterwards, cell viability was investigated to identify the best incubating time of HHDPCs and plasmaconcentration. Furthermore, the expression levels of versican and alkaline phosphatase (ALP) in PRP- or PPP-treated HHDPCs were checked.

Materials and methods

Sample preparation

A total of 8 healthy volunteers (8 males, mean age = 24.9 years) were enrolled and provided

their inform consent. About 10 ml whole blood was drawn from median cubital vein of each volunteer and stored in centrifuge tubes containing 3.2% sodium citrate (vol/vol = 10:1, BD). Then, PRP and the PPP were prepared using 2-step centrifugation method as described previously [15]. After PRP was activated by adding 1000 U bovine thrombin and 0.2 ml of 10% $CaCl_2$, it was placed at room temperature for about 10 min and then recentrifuged (3200 r/min, 5 min, r = 13.5 cm). Finally, the supernatant was collected and stored at -80°C.

HHDPCs cultivation

The second-generation HHDPCs were purchased from Shanghai Huzheng Industrial CO. LTD. The cell were resuspended in the mixture of RPMI 1640 medium (GIBCO), 10% fetal bovine serum (FBS, GIBCO) and 1% double antibody (GIBCO). Followed bythe suspension was cultivated at 37°C in a humidified 5% CO_2 incubator (Thermo). Then, the second-generation HHDPCs were spread on Petri dish for passage. When 80%~90% of the Petri dish were covered, HHDPCs were digested with pancreatin (GIBCO). After centrifugation, the supernatant was removed. Subsequently, the HHDPCs were added with the frozen stock solution made from 50% RPMI 1640 medium (GIBCO), 40% FBS (GIBCO) and 10% dimethyl sulfoxide (DMSO). Finally, the HHDPCs were preserved in a program frozen box.

MTT assay

The fourth- (p4) and eighth-generation (p8) HHDPCs were counted, and then each well of 96-well plates (ABI) was added with 1×10^4 cells. After the HHDPCs were starved for 24 h, their medium was replaced. In experimental group 1, the medium was replaced by medium containing 5% or 10% PRP. In experimental group 2, the medium was changed into medium containing equal PPP. Meanwhile, the medium in control groupwere replaced by normal medium. There were six repeatsfor each group. After HHDPCs being incubated for 24 h, 48 h and 72 h, 10 μl MTT reagent (0.5 mg/ml) was added to each well and thenthe 96-well plate was incubated at 37°C for 4 h. Subsequently, the medium of HHDPCs was replaced by 100 μl DMSO. Followed bythe plate was shook gently for 10 min to dissolvecrystals thoroughly. Finally, the optical densities (ODs) of HHDPCs solution in each well were measured at 570 nm by spectrophotometer (Merinton). Each assay was repeated for three times.

RNA extraction and first-strand cDNA synthesis

After the HHDPCs (p4 and p8) were counted, 2×10^5 cells were added into each well of 6-well plates (ABI) and starved for 24 h. In experimental group 1, the medium of HHDPCs was changed into medium containing 5% PRP. In experimental group 2, the medium was replaced by medium containing equal PPP. In control group, the medium were replaced by normal medium. Each group had two repeats. After incubation for 72 h, HHDPCs were collected for RNA extraction and the following western blot. Total RNA was extracted using Trizol-based procedure as described previously [16]. The purity and concentration of total RNA were measured using spectrophotometer (Merinton) and described by A260/A280. Using a reverse transcription kit (TaKaRa), reverse transcription experiment was performed for total RNA to synthesize first-strand cDNA. In addition, the synthesized cDNA was stored at -20°C.

Real-time quantitative PCR (qRT-PCR) analysis

The primers for qRT-PCR were quoted from the study of Yamauchi *et al*. [17]. Versican and ALP expression levels were evaluated using 100 ng/μl of cDNA template. The reaction systemcontained 10 μl SYBR Premix Ex Taq (2×, ABI), 1 μl cDNA, 1 μl forward primer (10 mM), 1 μl reverse primer (10 mM) and ddH_2O to reach a total volume of 20 μl. The reaction program was as follows: 50°C for 3 min, 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. After the results were obtained, a melting curve was created. The β-actin was used as reference gene, and all samples had three repeats.

Western blot analysis

Cell solution was drawn to prepare protein sample. The HHDPCs were washed with 2 ml precooled PBS and then digested by pancreatin. After being resuspended in 1 ml PBS, the HHDPCs were centrifuged at 600 g for 5 min at 4°C. Subsequently, the cells were dissolved by lysates (Sangon) containing PMSF (Sangon) and centrifuged at 12000 rpm for 10 min at 4°C. Using bicinchoninic acid (BCA), concentra-

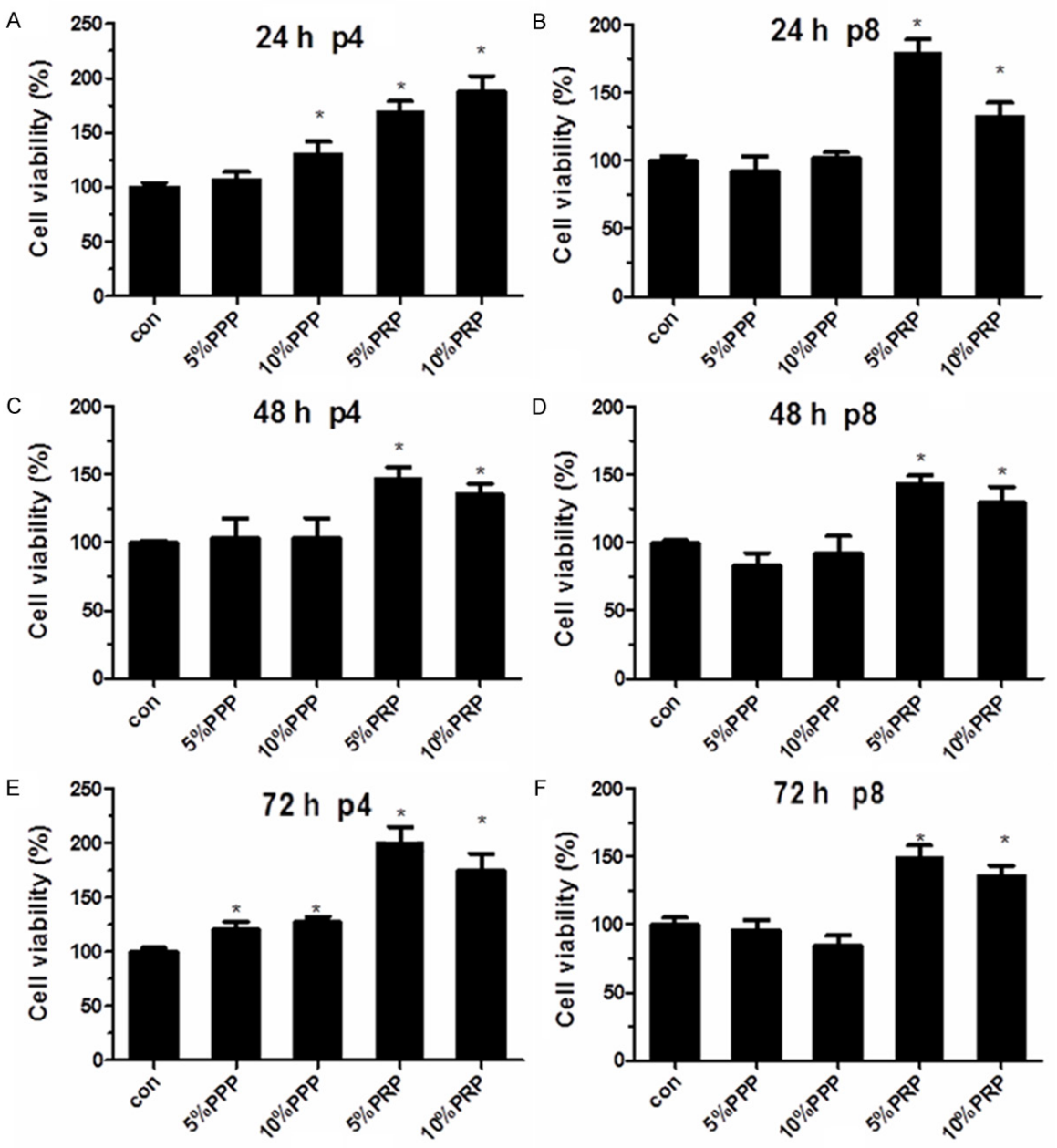

Figure 1. Cell viability investigated by MTT assays. A. The cell viability of the HHDPCs (p4) in 24 h group. B. The cell viability of the HHDPCs (p8) in 24 h group. C. The cell viability of the HHDPCs (p4) in 48 h group. D. The cell viability of the HHDPCs (p8) in 48 h group. E. The cell viability of the HHDPCs (p4) in 72 h group. F. The cell viability of the HHDPCs (p8) in 72 h group. The error bars stand for standard deviations from three samples. *$P < 0.05$ vs. the control group.

tions of the protein were determined. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% stacking gel and a 10% separating-gel. Afterwards, the separated proteins were transferred to polyvinylidene fluoride membranes (Merck Millipore). The membranes were blocked with sealing solution (containing 5% non-fat milk) for 1 h at room temperature, and incubated with primary antibodies (anti-versican, 1:50 dilution, Boster and anti-ALP, 1:1000 dilution, ABSCI) for a night at 4°C. After being washed in 1×TBST for three times, the membranes were incubated by secondary antibody (1:10000 dilution, Jackson) for 1 h. Then, the membranes were washed in 1×TBST for three

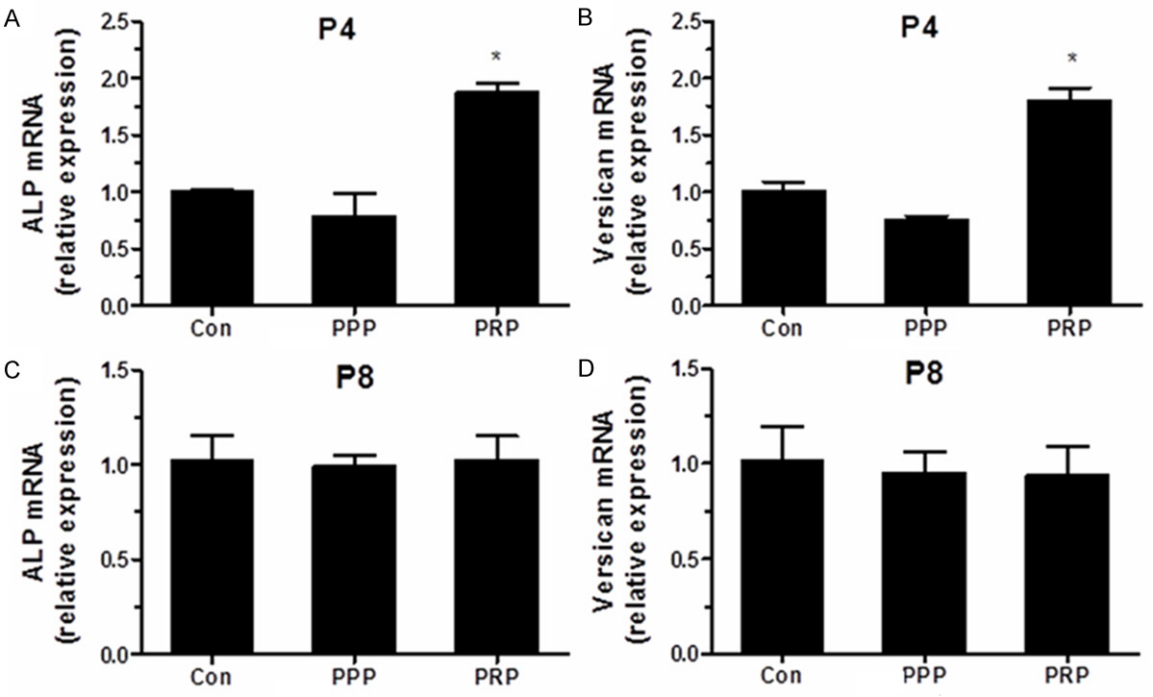

Figure 2. Expression of ALP and versican in HHDPCs detected by real-time quantitative PCR. A. The mRNA level of ALP in the HHDPCs (p4). B. The mRNA level of versicanin the HHDPCs (p4). C. The mRNA level of ALP in the HHDPCs (p8). D. The mRNA level of versicanin the HHDPCs (p8). The error bars stand for standard deviations from three samples. *P < 0.05 vs. the control group.

times again. Using ECL chemoluminescence kit (Merck Millipore), the membranes were developed in gel document (Bio-Rad).

Statistical analysis

After qRT-PCR being performed, the mRNA levels of ALP and versicanwere calculated bythe $2^{-\Delta\Delta Ct}$ method [18]. The data were analyzed by SPSS 13.0 (SPSS Inc) and exhibited as mean ± standard error of mean (SEM). A P < 0.05 was used as the cut-off criterion.

Results

The cell viability of HHDPCs treated by PRP and PPP

To evaluate the cell viability of HHDPCs treated by PRP and PPP, MTT assays were performed. Compared with the control group, the cell viability in the groups treated with PRP were significantly higher, especially for the HHDPCs (p4) in 24 h (**Figure 1A**) and 72 h groups (**Figure 1E**) (P < 0.05). Meanwhile, 10% PPP could significantly promote cell viability of the HHDPCs (p4) in 24 h (**Figure 1A**) and 72 h (**Figure 1E**) groups (P < 0.05). And 5% PPP could also significantly accelerate cell viability of the HHDPCs (p4) in 72 h group (**Figure 1E**) (P < 0.05). However, the effect of 10% PRP on HHDPC viability was less than that of 5% PRP.

Analysis of ALP and versican expression

PRP could significantly increase the expression levels of ALP (**Figure 2A**) and versican (**Figure 2B**) in HHDPCs (p4) compared with the control group (P < 0.05). However, PRP and PPP had no significant effect on ALP and versican expression in HHDPCs (p8) and HHDPCs (p4 or p8), respectively.

Western blot analysis

Compared with the control group, PRP could obviously increase the protein levels of ALP and versican in HHDPCs (p4) (**Figure 3A**). However, PRP had no remarkable influence on the protein levels of ALP and versican in HHDPCs (p8). Moreover, PPP had obvious impact on the ALP expression in HHDPCs (p4) (**Figure 3A**), but had no prominent effect on the versican expression in HHDPC cells (p4 or p8) and the ALP expression in HHDPCs (p8).

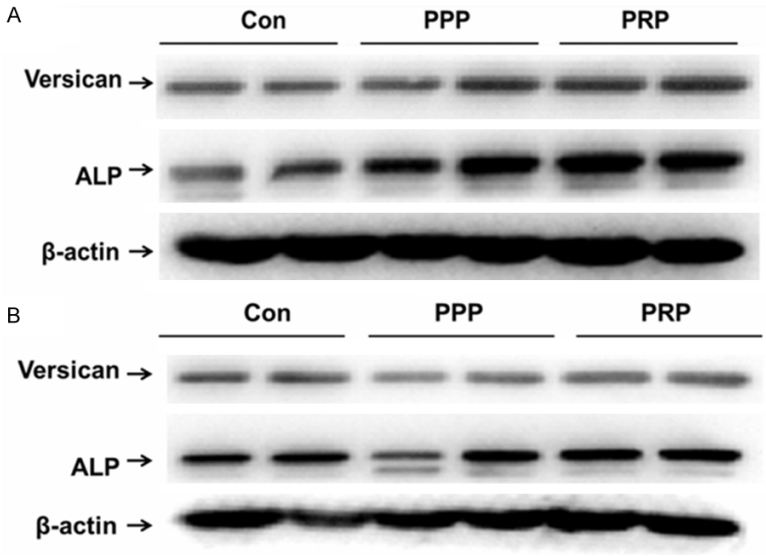

Figure 3. Protein levels of ALP and versican in HHDPCs investigated using western blot. A. The protein level of ALP and versican in HHDPCs (p4). B. The protein level of ALP and versican in HHDPCs (p8).

Discussion

In this study, both PRP (5% and 10%) and PPP (5% and 10%) could significantly promote the cell viability of the HHDPCs (p4) in 72 h group. However, the effect of 10% PRP on HHDPC viability was less than that of 5% PRP. The 10% PRP did not provide a better environment for HHDPCs viability, indicating that 10% PRP might mainly contribute to HHDPC differentiation. The results of the three assays were different in HHDPCs (p4) and HHDPCs (p8), suggesting that the viability of HHDPCs (p8) was worse than that of HHDPCs (p4).

Versican, which belongs to the family of large aggregating chondroitin sulfate proteoglycan, functions in cell proliferation, differentiation, migration and adhesion [19, 20]. For example, versican V1 isoform can promote cell proliferation, control cell cycle progression, and protect the cells from apoptosis [21]. Through functioning in cell adhesion, as well as matrix assembly and structure, versican is involved in hair follicle morphogenesis and cycling [22]. The expression of ALP may be associated with dermal papilla (DP)'s functions in hair formation and structural reconstruction of bulbar during hair follicle regeneration [23]. Results of qRT-PCR showed that PRP could significantly increase the mRNA levels of ALP and versican in HHDPCs (p4) compared with the control group. This was in accordance with the results of western blot, which showed obviously increased protein levels of ALP and versican in PRP-treated HHDPCs (p4). Thus, PRP hadan effect of promoting cell proliferation and differentiation on HHDPCs as indicated by the versican and ALP expression.

Platelet-released growth factors, including platelet-derived growth factor (PDGF), platelet-derived epidermal growth factor (PDEGF), transforming growth factor (TGF), insulin growth factor-1 (IGF-1), platelet-derived angiogenesis factor (PDAF) and platelet factor-4 (PF-4), can be advantageous for cell proliferation, cell differentiation, and even tissue regeneration [24, 25]. Additionally, some other factors including interleukin-1 (IL-1), epithelial cell growth factor (ECGF) and basic fibroblast growth factor (bFGF) have been found in the a-granules of the platelets [26-28]. As an antilogoussource of growth factors, PRP might stimulate cell proliferation and differentiation of HHDPCs through the factors in it.

Previous study reports that PRP can help DPC proliferation, promote hair growth and be used as a promising therapeutic tool [29]. Also, dalteparin and protamine microparticles (D/P MPs) can carry growth factors in PRP effectively, and PRP-containing D/P MPs (PRP & D/P MPs) can better contribute to hair growth [30]. Through chamber assay, Miao et al. find that PRP can help hair formation and induce hair follicle reconstitution in vivo [13]. PRP is reported to be able topromote hair growth in patients with alopecia through DPC proliferation, as well as keratinocyte growth factor (KGF) and B-cell leukemia/lymphoma 2 (BCl-2) up-regulation [31]. These are in keeping with our results. The increase of HHDPC proliferation contributes to clinical applications of HHDPCs in hair follicle regeneration. Thus, our research provided an experimental evidence for the clinical application of PRP as a potential tool to promote HHDPC proliferation.

In conclusion, the results of our study indicated that PRP can stimulate HHDPCs viability. Besides, 5% PRP could significantly increase the ALP and versican expression in both mRNA and protein levels in HHDPCs (p4). However, this is an in vitro study, and the factors that play important roles in PRP-induced cell viability haven't been identified. There are many growth factors in PRP; hence, the identification of the exact factors inducing proliferation will cost much time and effort.

Acknowledgements

This study was supported by the Science and Technology Projects Fund of Hangzhou City (Grant No. 20130633B04).

Disclosure of conflict of interest

None.

Address correspondence to: Ming Jia, Department of Plastic Surgery, Hangzhou First People's Hospital, Nanjing Medical University, 261 Huansha Road, Hangzhou 310006, Zhejiang, P.R China. Tel: +86-57156006533; Fax: +86-57187914773; E-mail: mingjiamj@163.com

References

[1] Stenn K and Paus R. Controls of hair follicle cycling. Physiol Rev 2001; 81: 449-494.
[2] Ito M, Yang Z, Andl T, Cui C, Kim N, Millar SE and Cotsarelis G. Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. Nature 2007; 447: 316-320.
[3] Gilhar A and Kalish RS. Alopecia areata: a tissue specific autoimmune disease of the hair follicle. Autoimmun Rev 2006; 5: 64-69.
[4] Kulick D, Hark L and Deen D. The bariatric surgery patient: a growing role for registered dietitians. J Am Diet Assoc 2010; 110: 593-599.
[5] Zhong S, Zhang Y and Lim C. Tissue scaffolds for skin wound healing and dermal reconstruction. Wiley Interdiscip Rev Nanomed Nanobiotechnol 2010; 2: 510-525.
[6] Akalin C, Kuru S, Barlas AM, Kismet K, Kaptanoglu B, Demir A, Astarci HM, Ustun H and Ertas E. Beneficial effects of Ankaferd Blood Stopper on dermal wound healing: an experimental study. Int Wound J 2014; 11: 64-68.
[7] Oshima H, Inoue H, Matsuzaki K, Tanabe M and Kumagai N. Permanent restoration of human skin treated with cultured epithelium grafting-wound healing by stem cell based tissue engineering. Hum Cell 2002; 15: 118-128.
[8] Eppley BL, Woodell JE and Higgins J. Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing. Plast Reconstr Surg 2004; 114: 1502-1508.
[9] Kocaoemer A, Kern S, Klueter H and Bieback K. Human AB serum and thrombinbinor analysis from platelet-rich plasma: implications for wound healing. Plastic and reconstructive surgery 2004; 114: 1502-1508. 1se tissue. Stem Cells 2007; 25: 1270-1278.
[10] Vogel JP, Szalay K, Geiger F, Kramer M, Richter W and Kasten P. Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and in vivo bone formation in calcium phosphate ceramics. Platelets 2006; 17: 462-469.
[11] Mishra A, Tummala P, King A, Lee B, Kraus M, Tse V and Jacobs CR. Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation. Tissue Eng Part C Methods 2009; 15: 431-435.
[12] Reese RJ. Autologous platelet rich plasma (PRP): what do we know? Important concepts relevant to hair restoration surgery. In Hair Transplant Forum Int 2010.
[13] Miao Y, Sun YB, Sun XJ, Du BJ, Jiang JD and Hu ZQ. Promotional Effect of Plateleteletelevasma on Hair Follicle Reconstitution in vivo. Dermatol Surg 2013; 39: 1868-1876.
[14] Jahoda CA, Horne KA and Oliver RF. Induction of hair growth by implantation of cultured dermal papilla cells. Nature 1984; 311: 560-2.
[15] Franco D, Franco T, Schettino AM, Tavares Filho JM and Vendramin FS. Protocol for obtaining platelet-rich plasma (PRP), platelet-poor plasma (PPP), and thrombin for autologous use. Aesthetic Plast Surg 2012; 36: 1254-1259.
[16] Meng L and Feldman L. A rapid TRIzolIzols Filho JM and Vendramin FS. ee RNA extraction from Arabidopsis siliques and dry seeds. Biotechnol J 2010; 5: 183-186.
[17] Yamauchi K and Kurosaka A. Inhibition of glycogen synthase kinase-3 enhances the expression of alkaline phosphatase and insulin-like growth factor-1 in human primary dermal papilla cell culture and maintains mouse hair bulbs in organ culture. Arch Dermatol Res 2009; 301: 357-365.
[18] Arocho A, Chen B, Ladanyi M and Pan Q. Validation of the 2-ΔΔCt calculation as an alternate method of data analysis for quantitative PCR of BCR-ABL P210 transcripts. Diagn Mol Pathol 2006; 15: 56-61.
[19] Cattaruzza S, Schiappacassi M, Ljungberg-Rose Å, Spessotto P, Perissinotto D, Mörgelin M, Mucignat MT, Colombatti A and Perris R. Distribution of PG-M/Versican Variants in Human Tissues andde Novo Expression of Iso-

form V3 upon Endothelial Cell Activation, Migration, and Neoangiogenesis in Vitro. J Biol Chem 2002; 277: 47626-47635.
[20] Cattaruzza S, Schiappacassi M, Kimata K, Colombatti A and Perris R. The globular domains of PG-M/versican modulate the proliferation-apoptosis equilibrium and invasive capabilities of tumor cells. FASEB J 2004; 18: 779-781.
[21] Sheng W, Wang G, Wang Y, Liang J, Wen J, Zheng PS, Wu Y, Lee V, Slingerland J, Dumont D, Yang BB. The roles of versican V1 and V2 isoforms in cell proliferation and apoptosis. Mol Biol Cell 2005; 16: 1330-1340.
[22] du Cros DL, LeBaron RG and Couchman JR. Association of versican with dermal matrices and its potential role in hair follicle development and cycling. J Invest Dermatol 1995; 105: 426-431.
[23] Iida M, Ihara S and Matsuzaki T. Hair cycleycletion of versican with dermal matrices and its potential role in hair fopithelium in mouse vibrissal follicles. Dev Growth Differ 2007; 49: 185-195.
[24] Dolder JVD, Mooren R, Vloon AP, Stoelinga PJ and Jansen JA. Platelet-rich plasma: quantification of growth factor levels and the effect on growth and differentiation of rat bone marrow cells. Tissue Eng 2006; 12: 3067-3073.
[25] Kakudo N, Minakata T, Mitsui T, Kushida S, Notodihardjo FZ and Kusumoto K. Proliferation-promoting effect of platelet-rich plasma on human adipose-derived stem cells and human dermal fibroblasts. Plast Reconstr Surg 2008; 122: 1352-1360.
[26] Marx RE. Platelet-rich plasma: evidence to support its use. J Oral Maxillofac Surg 2004; 62: 489-496.
[27] Aghaloo TL, Moy PK and Freymiller EG. Investigation of platelet-rich plasma in rabbit cranial defects: a pilot study. J Oral Maxillofac Surg 2002; 60: 1176-1181.
[28] Tözüm TF and Demiralp B. Platelet-rich plasma: a promising innovation in dentistry. J Can Dent Assoc 2003; 69: 664.
[29] Li ZJ, Choi HI, Choi DK, Sohn KC, Im M, Seo YJ, Lee YH, Lee JH and Lee Y. Autologous platelet-rich plasma: a potential therapeutic tool for promoting hair growth. Dermatol Surg 2012; 38: 1040-1046.
[30] Takikawa M, Nakamura S, Nakamura S, Ishirara M, Kishimoto S, Sasaki K, Yanagibayashi S, Azuma R, Yamamoto N , Kiyosawa T. Enhanced effect of platelet- rich plasma containing a new carrier on hair growth. Dermatol Surg 2011; 37: 1721-1729.
[31] Cervelli V, Garcovich S, Bielli A, Cervelli G, Curcio BC, Scioli MG, Orlandi A and Gentile P. The Effect of Autologous Activated Platelet Rich Plasma (AA-PRP) Injection on Pattern Hair Loss: Clinical and Histomorphometric Evaluation. Biomed Res Int 2014; 2014: 760709.