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Original article

Platelet-rich plasma, platelet-rich fibrin, and enamel matrix derivative for oral mucosal wound healing

J. Vokurka[1,2*†], F. Hromcik[1,2*], M. Faldyna[3], E. Gopfert[3], M. Vicenova[3], L. Pozarova[2,4], L. Izakovicova Holla[1,2]

[1] Clinic of Dentistry, St. Anne's University Hospital, Pekarska 53, 656 91 Brno, Czech Republic
[2] Faculty of Medicine, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic
[3] Veterinary Research Institute, Brno, Palackeho tr. 1946/1, 612 42 Brno, Czech Republic
[4] First Department of Pathological Anatomy, St. Anne's University Hospital, Pekarska 53, 656 91 Brno, Czech Republic

Abstract

Different approaches to enhance healing of hard or soft tissues include the use of cytokines and growth factors to modify cellular behaviour. Numerous growth factors are found in autologous blood concentrates – platelet-rich plasma (PRP) and platelet-rich fibrin (PRF). Enamel matrix derivative (EMD) may improve tissue healing via amelogenins. Bilayered collagen matrix (CM) is used for soft tissue augmentation.

The aim of the present study was to assess potential benefits of PRP, PRF and EMD in combination with bilayered collagen matrix or CM alone in treatment of oral mucosal defects in rabbits.

Twenty-seven New Zealand white rabbits were included in this randomized controlled trial. Artificial oral mucosal defects were treated with one of these five approaches: PRP+CM, PRF+CM, EMD+CM, CM alone, or left untreated as a negative control - CO. The animals were euthanized 1 day, 7 days, or 28 days after surgery and necropsies were harvested. Histological and molecular biological analyses were performed.

All defects were healed by day 28. No differences between PRP+CM, PRF+CM, CM alone and CO groups were recorded at any time point. Slower angiogenesis and a higher presence of inflammatory infiltrate were observed in the EMD+CM group 28 days after surgery. Molecular biological analyses did not reveal any statistically significant changes.

In conclusion, no improvement in mucosal healing of wounds covered with a collagen membrane and PRP, PRF, or EMD was observed, compared with CM alone or untreated controls.

Key words: platelet-rich plasma, platelet-rich fibrin, wound healing, dental enamel proteins, artificial membranes

Correspondence to: L. Izakovicova Holla, e-mail: holla@med.muni.cz, tel.: +420 543 183 406
[*] These authors contributed equally to this work.

Introduction

Mucosal healing is in the centre of attention of periodontology, oral surgery, and implantology. Autogenous platelet concentrates such as platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) are used to enhance mucosal and osseous healing via growth factors released from granules of the platelets, causing faster inflammatory response and enhancement of healing process (Marx et al. 1998). Enamel matrix derivative (EMD; Emdogain®, Straumann AG, Switzerland) is also supposed to influence scar formation and healing outcome. PRP, PRF or EMD are used in everyday practice of many dentists although the efficacy of these products has not been directly compared yet.

Growth factors (GFs) released from platelets' alpha granules are able to promote fibroblast proliferation, increase tissue vascularity, and accelerate bone repair (Marx et al. 1998, Del Corso et al. 2012, Vokurka, Fassmann and Izakovičová Hollá 2015). Numerous platelet concentrates made from autologous blood have been developed in the past 20 years, but only limited attention has been paid to the comparison of their efficacy.

Platelet-rich plasma enhances healing of both bony and mucosal defects, especially in terms of leukocyte attraction, differentiation, and replication of stem cells (Dohan Ehrenfest 2010, Albanese et al. 2013). Nevertheless, a PRP has a strong stimulant effect on capillary regeneration in wound healing during the early stages of wound healing. (Lindeboom et al. 2007, Keskiner et al. 2014). Platelet-rich fibrin has been introduced as a second-generation product from autologous blood source (Moraschini and Barboza 2015, Miron and Choukroun 2017). Although it does not contain as high concentration of GFs compared to PRP, its ability to incorporate autologous GFs into a fibrin mesh is believed to boost the healing process (Davis et al. 2014, Del Fabbro et al. 2014). Moreover, unlike PRP, it is cheap and easy to prepare.

Enhancement of healing has been described also for enamel proteins extracted from porcine foetuses, the main components of EMD. These proteins are able to accelerate formation of granulation tissue, induce proliferation of fibroblasts, or provoke production and release of growth factors (Bosshardt 2008). Currently, no study comparing EMD with blood plasma derivatives in soft tissue healing is known to the authors.

Collagen matrix (CM) is used for augmentation of soft tissues in periodontology and implant dentistry. For regenerative procedures, CM is available as a double layer porcine collagen membrane which enhances the soft tissues around both teeth and implants (Mucograft®, Geistlich AG, Switzerland). The capacity of CM as a carrier for autologous blood derivatives must be verified.

The aim of the present study was to compare healing of artificial defects treated with PRP, PRF, and EMD together with collagen matrix or CM alone in a rabbit model.

Materials and Methods

Ethical statement

The experiment was performed in compliance with the Act No. 246/1992 of Collection of the Czech National Council on the protection of animals against cruelty, and with the agreement of the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic.

The study was performed according to ARRIVE guidelines for reporting in vivo animal research (Kilkenny et al. 2010).

Study design

This study presents an animal experiment conducted in a split-mouth fashion, in the randomized complete block design. Five defects were created in each of 27 animals. Then, every defect was assigned randomly (upon drawing lots) to one of the five treatment options. Thus, a total of 135 sites were divided into 5 groups. Before the interventions, the animals were randomly divided into 3 time-point groups according to the day of necropsy (Day 1, Day 7, Day 28). None of these details were known to the pathologist during the evaluation of harvested samples.

Animal model used

In the study, New Zealand white rabbits (*Oryctolagus cuniculus*), provided by a local accredited producer, were used. Prior to and during the experiment, the animals were housed under standard conditions at the Veterinary Research Institute (Brno, Czech Republic) and fed daily with no limitations. No adverse events occurred. All the animals thrived during the experimental period.

PRP preparation

Preparation protocol of PRP in our study was as follows: Ten millilitres of peripheral venous blood from *vena auricularis* was drained into a testing tube with 50 IU of heparin. The sample was centrifuged for 10 min with 400 g (centrifugal force) without brake at 21°C. Buffy coat and the superior layer of erythrocytes were collected and transferred into a new tube

Table 1. Scoring system according to Abramov (2007). The scheme used for a semiquantitative evaluation of re-epithelialization, level of angiogenesis, the presence of granulation tissue, quantity of collagen and macrophages, acute and chronic inflammatory infiltrate.

Re-epithelialisation	Angiogenesis	Granulation tissue	Collagen (after staining with green trichrome)	Macrophages (marked with CD 68)		Acute inflammatory infiltrate	Chronic inflammatory infiltrate
0	0	0 (no fibroblasts)	0	0-12	1a	0	0
1 (partial)	1 (>5 vessels/HPF)	1 (few fibroblasts)	1	13-25	1b	1	1
2 (complete, but immature or thin)	2 (6-10 vessels/HPF)	2 (more fibroblasts)	2	26-37	2a	2	2
3 (complete and mature)	3 (>10 vessels/HPF)	3 (most fibroblasts)	3	38-50	2b	3	3
				>50	3		

and centrifuged for 10 min with 500 g at 21°C. After the centrifugation, 1.8 ml of PRP was obtained and immediately used during the surgery.

PRF preparation

For PRF preparation, 8 ml of peripheral venous blood was spun in a glass test tube with no added anticoagulant in a PC02 centrifuge (Process, Nice, France) for 14 minutes at 1500 rpm. The PRF clot was then removed from the tube and inserted into a PRF box for further use.

Intervention

Rabbits were anaesthetized with propofol 8-15 mg/kg IV for surgical creation of five artificial round defects with a diameter of 5 mm in the maxillary region using a mucotome and a 15C blade. The defects were filled either with CM alone, CM with PRP, CM with PRF, CM with EMD, or left untreated as the negative control (CO). The animals were divided randomly into three groups. The first group was sacrificed 1 day after surgery, the second after 7 days, and the third after 28 days. Subsequently, necropsies from the studied region were harvested and preserved for histological, immunohistochemical and molecular biological analysis.

Histopathological assessment

For light microscopy, the samples were dyed with haematoxylin and eosin (H&E) staining. Special staining Masson's trichrome was used for the detection of collagen fibres. The specimens were evaluated at magnification of x400. New vessels and macrophages were counted in the specific area. In the specimens, the macrophages were detected immunohistochemically by use of Monoclonal Mouse Anti-Rabbit Macrophage Clone RAM11, isotope IgG1 (DAKO, Glostrup, Denmark). The pathologist rated semiquantitatively according to Table 1.

Molecular biological assessment

Samples for molecular biological analyses were lysed in TRI Reagent RT (Molecular Research Centre, Cincinnati, OH, United States). Total RNA with elution volume of 15 µl was obtained using the combination of 4-bromoanisole phase separation followed by silica-based RNeasy purification (Qiagen, Hilden, Germany). mRNA was specifically reverse-transcribed using M-MLV reverse transcriptase system (Invitrogen, Paisley, UK) in the presence of oligo-dT primer. cDNA was diluted 5x and 0.5 µl used in qPCR. In qPCR analysis, RNA expression was quantified in triplicate reactions in a final volume of 3 µl in 384-well plates using QuantiTect SYBR Green PCR master mix (Qiagen, Hilden, Germany) following the manufacturer's recommendations. qPCR reactions were prepared with the assistance of Nanodrop II liquid dispenser (Innovadyne Technologies, Rohnert Park, CA, United States). qPCR was performed under the following conditions: denaturation (95°C for 15 min) and 45 amplification cycles (95°C for 15 s, 58°C for 30 s and 72°C for 30 s). Resulting melting curves were analysed to test the product specificity. Each couple of primers (Generi Biotech, Hradec Kralove, Czech Republic) at 10 *p*mol was used per reaction. Primers specific to eight target genes, coding for cytokines with pro- and anti-inflammatory properties, and three reference genes (Table 2) were used for simultaneous measurements of gene expression activity. From the obtained data, relative expression of each target gene was calculated according to the formula [1/(2target gene Ct)]/[1/(2reference gene Ct)].

Statistical analysis

The sample size calculation in this exploratory experiment was based on the "resource equation" (Mead 1988). With nine animals and five defects/interventions each, designed in nine blocks, we got error degrees of freedom = 33 for each of the three time points (calculated for ANOVA test). Although this num-

Table 2. Primers used for molecular biologic assessment of inflammatory response.

Gene	Primer sequence (3′- 5′)	Function
IL-1β	*F*: ACCAACAAGTGGTGTTCTCCATGA	Interleukin 1β, pro-inflammatory cytokine
	R: TTTCATCACGCAGGACAGGTACA	
TNF-α	*F*: CTCTGCCTCAGCCTCTTCTCTT	Tumor necrosis factor α, pro-inflammatory cytokine
	R: AGGTTGTTTGGGGACTGCTCT	
IL-17	*F*: ACCACATGAACTCTGTCCCAATC	Interleukin 17, proinflammatory cytokine, chemokine, production of antibacterial peptides
	R: CCTACAGCCACCAGCATCTTC	
MMP9	*F*: CACTGGGCTTGGATCACTCCTC	Matrix metalloproteinase 9, proinflammatory mediator, destruction of tissue
	R: GGGTTAGGACCATATAGATGCTGGA	
IL-10	*F*: TTCTGTGCCTGACCACACTTTC	Interleukin 10, antiinflammatory cytokine
	R: CTAGGAGTCTCTGGAACACTCGG	
TGF-β	*F*: TTCCCCTCCGAAAATGCCATCC	Transforming growth factor β, antiinflammatory cytokine
	R: CACTCTGGCTTTTGGGTTCTGC	
VEGF	*F*: GCTTCTTGTCTCTGGCGTGTTC	Vascular endothelial growth factor, antiinflammatory cytokine, vascularization
	R: CCTACATAAGCCTTGGCCTCCTC	
TIMP1	*F*: GTTTCTCATCGCTGGACAACTGC	Tissue inhibitor of metalloproteinases, antiinflammatory mediator, inhibitor of MMP9
	R: ACGAAACTGCAAGTCGTGATGTG	
HMBS	*F*: CAGCCATGAAGGATGGGCAGCTGTAC	Reference gene
	R: TGCTGGCCTGCATGGTCTCTTGC	
HPRT	*F*: TGAAACTGGAAAAGCAAATACAAAG	Reference gene
	R: CGATGTCAATGAGACTCCTGATG	
GAPDH	*F*: GAATCCACTGGCGTCTTCAC	Reference gene
	R: CGTTGCTGACAATCTTGAGAGA	

ber exceeds an expected optimum of less than 20 error degrees of freedom, we considered this number at the beginning of the experiment. Thus, we avoided imbalances and less efficient statistics with ordinal (histopathologic scores) data caused by potential drop-outs during the experiment.

Molecular biological data was logarithmically transformed to achieve an approximately normal distribution. The treatment groups were compared using ANOVA test with an adjustment for complete block design, the comparison was performed separately for each time point (TP1, TP7). The data was summarized in plots as means with standard errors for the respective groups with accompanying statistical data.

ANOVA was used for the evaluation of histopathological score data summarized for each time point (TP1, TP7, TP28) using bar plots. The significance level alpha equalled to 0.05. The analyses were performed using SAS 9.3. and R (R Core Team 2019).

Results

Histopathological observation

At day 1 (Fig. 1), histological observation revealed an early defect with the presence of œdema, necrotic debris, haemorrhages, fibrin, and dense acute inflammatory infiltrate with a predominance of neutrophil granulocytes. The wounds were without signs of re-epithelialization. At day 7 (Fig. 2), proliferating granulation tissue filling the empty space after phagocytosis of necrotic tissue was detected around the defect. A mixed, predominantly acute inflammatory cellularization with a high number of neutrophil granulocytes and sporadic lymphocytes was present. An incipient re-epithelialization at wound margins was occasionally seen. At day 28 (Fig. 3), complete re-epithelialization of the wound was observed, the wound was overlaid with a focally immature squamous epithelium. A scar and chronic inflammatory

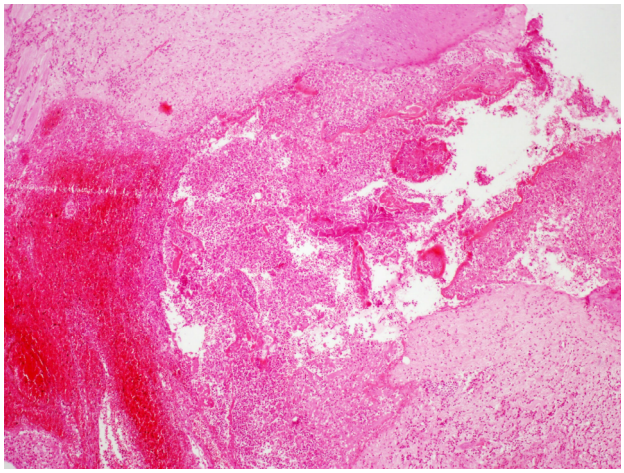

Fig. 1. Artificial defect, day 1. Defect with the presence of a dense acute inflammatory infiltrate with a predominance of granulocytes, filled with erythrocytes, neutrophil granulocytes and necrotic debris [haematoxylin-eosin staining (H&E), x200].

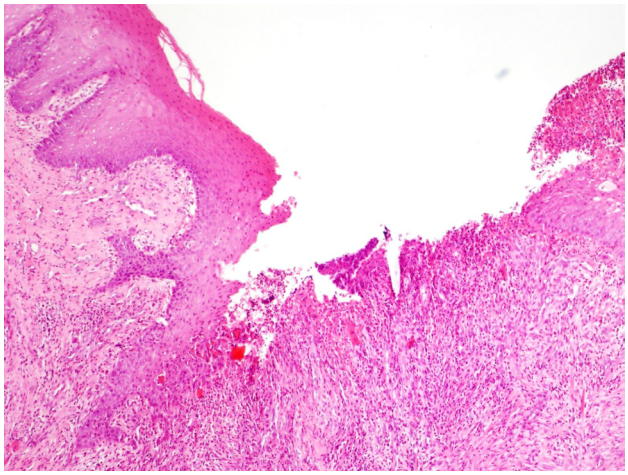

Fig. 2. Artificial defect, day 7. Defect with noticeable incipient re-epithelialization, filled with inflammatory infiltrated granulation tissue and covered with cell debris (H&E, x200).

infiltrate with lymphocytes and plasmocytes are filling the defect.

Although changes of histopathological parameters were easily noticeable at all time points (Fig. 4), no statistically significant differences between the study groups were found. Higher mean scores of angiogenesis and chronic inflammatory infiltrate were recorded only in the EMD group at day 28.

Molecular biological assessment

Similar to the results of the histopathological examination, the results of molecular biological analyses did not reveal any statistically significant changes among the treatments (Fig. 5).

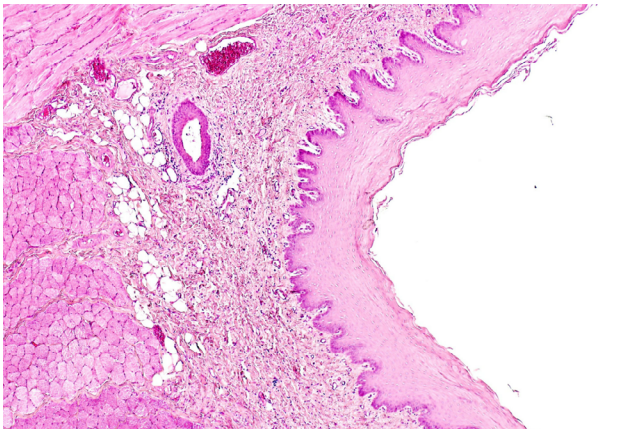

Fig. 3. Artificial defect, day 28. Complete re-epithelialisation of the wound, subepithelial granulation tissue transformed into the scar with a dense network of collagen fibres (H&E, x40).

Discussion

Many studies have confirmed the advantages of the use of PRP or PRF for the treatment of periodontal defects, single and multiple gingival recession, periimplantitis, medication-related osteonecrosis of jaws, and various other conditions (Del Fabbro et al. 2014, Del Fabbro et al. 2015a,b).

Some studies have shown positive effect of PRP and PRF during early stages of healing (Lindeboom et al. 2007, Suttapreyasri and Leepong 2013) whilst others have not confirmed such conclusions (Jankovic et al. 2012, Keskiner et al. 2014). Our study did not confirm the improved efficiency of PRP or PRF with CM compared to CM without blood plasma derivatives.

Both PRP and PRF have also been tested for their content of vascular endothelial growth factor (VEGF) prepared from rabbit blood (Vokurka et al. 2016). In this study, VEGF was found at lower concentrations in PRF compared to PRP, other parameters did not differ.

Molecular biology did not show any relevant differences either. Only regarding vascularization, higher mean scores of angiogenesis were noted in the EMD group at day 28. It may be since the expression of mRNA for TIMP was the lowest, unfortunately without statistical significance, in the EMD group at day 7. In a rat model, Miron et al. (2014) observed significantly increased angiogenesis in the early phases of tissue regeneration in the animals treated with EMD. Our study showed opposite results with a slight non-significant negative effect of EMD on early phases of angiogenesis.

Collagen matrix was proved in augmentation of the volume of keratinized gingiva (Sanz et al. 2009) compared to connective tissue graft. Our results did not prove any additional benefit of PRP, PRF, or EMD together with CM in terms of soft tissue healing.

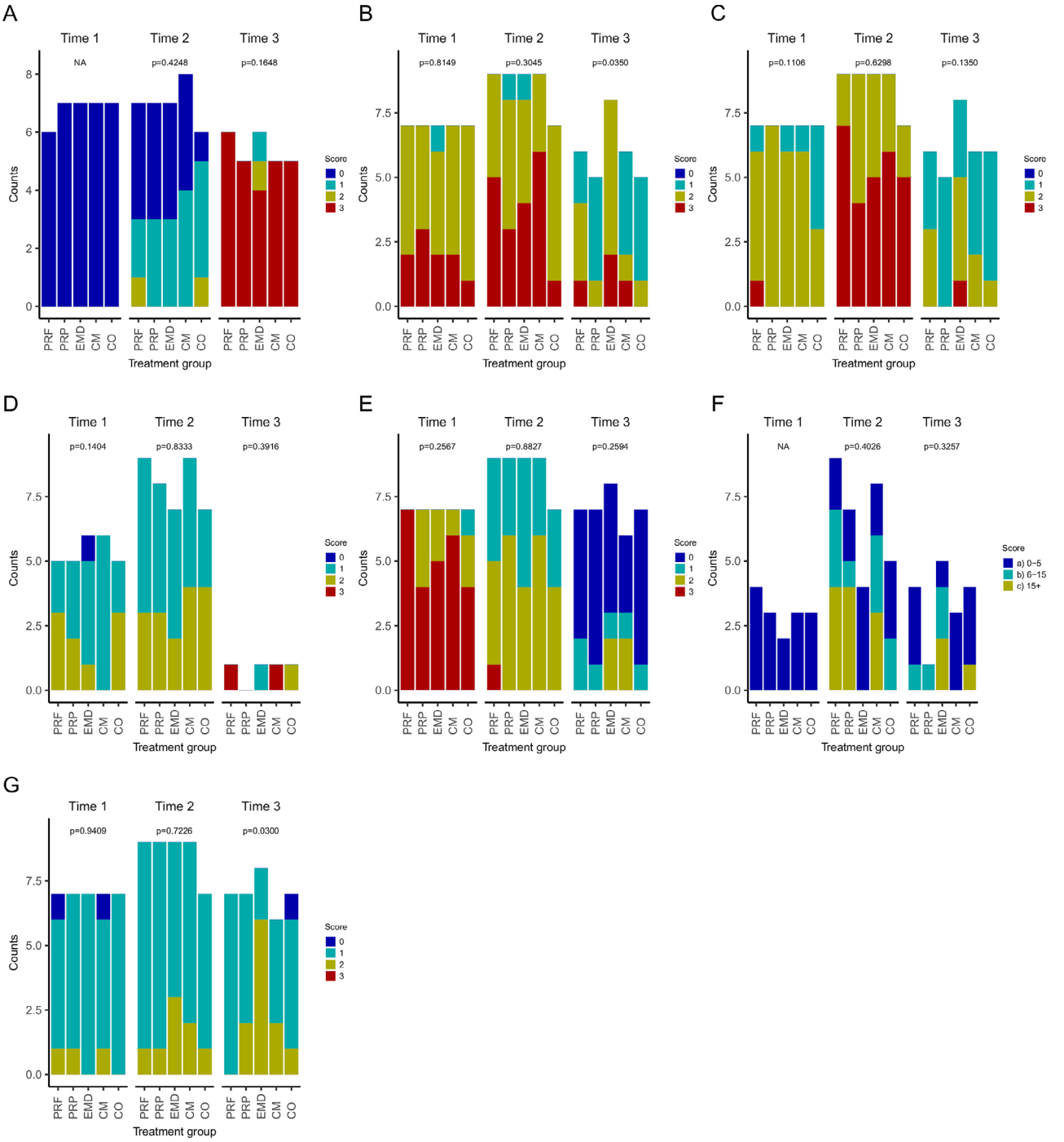

Fig. 4. Histopathological parameters. Presentation of statistical analysis of individual parameters at day 1 (Time 1), day 7 (Time 2) and day 28 (Time 3). (A) re-epithelialisation, (B) angiogenesis, (C) granulation, (D) collagen fibres, (E) macrophages, (F) acute inflammatory infiltrate and (G) chronic inflammatory infiltrate.

Conclusion

The positive effect of blood plasma derivatives (PRP or PRF) and EMD added to the collagen matrix was studied. This experimental study did not confirm any significant improvement of soft tissue healing when applying combination of CM with PRP, PRF, or EMD compared with CM alone or untreated controls.

Acknowledgements

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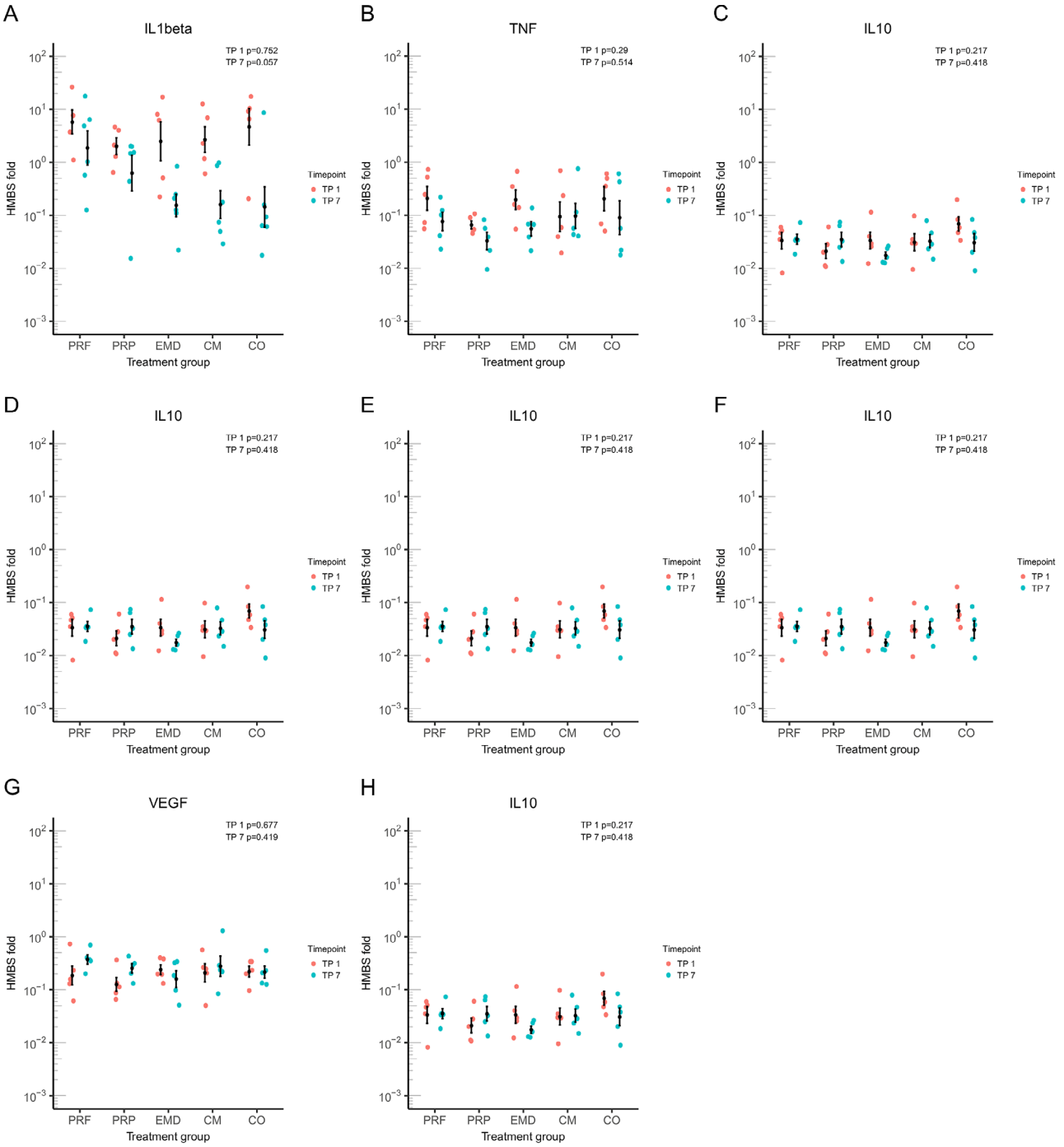

Fig. 5. Molecular biological analysis. Presentation of statistical analysis of the expression of pro- and anti-inflammatory genes at day 1 and day 7 of (A) IL-1β, (B) TNFα, (C) IL17, (D) MMP9, (E) IL-10, (F) TGF-β, (G) VEGF and (H) TIMP1.

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