

Platelet-rich Plasma and Platelet-poor Plasma Enhanced Attachment and Proliferation of Human Periodontal Cells to Diseased Root Surface

Qi LIU¹, Hai Yan DAI¹, Hai Yan ZHANG¹, P. Mark Bartold², Victor Marino²

Objective: To evaluate attachment and proliferation of human gingival fibroblasts (HGFs), human periodontal ligament fibroblasts (HPDLFs) and human osteoblasts (HOBs) on diseased root surfaces of human teeth demineralised with EDTA and subsequently treated with freshly prepared platelet-rich plasma (PRP) or platelet-poor plasma (PPP) from human blood.

Methods: HGFs, HPDLFs and HOBs were grown from tissue explants. Human whole blood from healthy subjects was collected to prepare PRP and PPP. The root surfaces of periodontitis-affected teeth were scaled, sectioned, and demineralised with EDTA and treated with PPP or PRP or none. The control group was neither demineralised nor treated with PPP or PRP. The cementum surfaces of root slices were cultured with HGFs, HPDLFs or HOBs for 24 or 48 h. Attachment and proliferation of cells were evaluated using cell count, scanning electron microscopy and MTT assay.

Results: The root surfaces of the control group demonstrated the least number of attached cells. Demineralisation alone or combined with PRP or PPP increased cell attachment and proliferation. Electron microscopic evaluation further confirmed that both PRP and PPP significantly enhanced cell attachment to the diseased root surfaces.

Conclusion: Pretreatment of diseased human root surfaces with EDTA, PRP or PPP enhances the attachment and proliferation of HGFs, HPDLFs and HOBs to the diseased root surface.

Key words: periodontal cells, attachment, platelet-rich plasma, platelet-poor plasma, demineralisation

A major goal of periodontal therapy is restoration of the damaged tissues to their original form and function. This requires regeneration of the destroyed periodontal connective tissues through formation of new cementum, new bone and new attachment to root surface¹. Various procedures have been advocated for the purpose of regenerating the periodontium, including the placement of bone grafts or bone substitutes, root

surface conditioning and demineralisation, or the use of organic or synthetic barrier membranes in guided tissue regeneration (GTR)²⁻⁶. More recently, biological principles have become the focus for regenerative techniques. The observation that platelet-rich plasma (PRP) contains high levels of platelet-derived growth factor and transforming growth factor- α and that platelet-poor plasma (PPP) contains high levels of fibrin led to the proposal that these polypeptide growth factors may have a beneficial effect by inducing periodontal regeneration⁷⁻¹². In recent years, many studies have confirmed that PRP and PPP are capable of aiding periodontal regeneration and modulating the proliferation of periodontally related cells *in vitro*¹³⁻¹⁵.

While cell attachment to the root surface and subsequent proliferation are considered to be important events in periodontal regeneration, the exact factors that medi-

1 Hospital of Dentistry, Zunyi Medical College, Zunyi, P.R. China

2 Colgate Australian Clinical Dental Research Centre, School of Dentistry, University of Adelaide, South Australia, Australia

Corresponding author: Dr Qi LIU, Hospital of Dentistry, Zunyi Medical College, Dalian Road, Zunyi 563003, P.R. China. Tel: 86-852-8609758. E-mail: liuqi1964@hotmail.com



ate these processes on periodontally diseased root surfaces are still unclear. Nonetheless, it is clear that cell attachment on pathologically diseased root surfaces is significantly altered compared to that occurring on healthy tooth roots¹⁶⁻¹⁹. Hence, some form of root surface detoxification followed by root surface treatment is considered to have the potential to enhance biocompatibility and, thus, potentially aid in periodontal regeneration²⁰. Therefore, for this study it was hypothesised that the addition of either PRP or PPP to previously scaled and chemically demineralised root surfaces would enhance cell attachment and proliferation.

Materials and Methods

Fibroblast cultures

Following institutional ethics approval, fibroblasts were obtained from human gingiva, periodontal ligament and alveolar bone as described previously^{21,22}. Tissue explants were cultured using Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) with 10% fetal calf serum (FCS) supplemented with penicillin (50 U/ml) and streptomycin (50 µg/ml). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The media was replaced every 2 or 3 days until confluence was reached. The human gingival fibroblasts (HGFs), human periodontal ligament fibroblasts (HPDLFs), and human osteoblasts (HOBs) used for this study were between their fifth and eighth passage in culture. These cell cultures had been obtained from explants taken with consent from extraction of healthy third molars, facelift, extraction of healthy premolar teeth for orthodontic reasons and alveolar surgery. To further identify these cells, tests were carried out to analyse cellular morphology, alkaline phosphatase activity and immunohistochemical protein expression.

Preparation of PRP and PPP

Human healthy whole blood (5 to 8 ml) was collected in 10 ml tubes coated with anticoagulant (10% sodium citrate–dextrose). Samples were centrifuged at 1300 rpm for 10 min to separate the red blood cells from the plasma in the supernatant. The supernatant was removed and centrifuged again for 10 min at 2000 rpm to prepare PPP and PRP²³. The PRP fraction was gently removed with a pipette and the preparation was always used fresh on the day of the experiment.

*Preparation of root slices for studies*²⁴

A total of 216 extracted periodontally involved human teeth were obtained. Before extraction, a pencil line was placed at the level of the gingival margin. Extractions were then performed without disturbing this area. Immediately following extraction, the tooth was rinsed and a groove was made at the pencil mark with a number 2 round bur. The test area was outlined using a number 2 bur, extending apically from the grooves at the line angles to the connective tissue attachment at the base of the pocket for identification of the portion of the tooth that was exposed to the pocket environment. All areas of the root surfaces were root planed with 15 strokes of a sharp 7/8 Gracey periodontal curette to simulate periodontal treatment of the root surface. Root specimens of approximately 4 mm × 4 mm were made of the diseased teeth to include the scribed area between the gingival margin and bottom of the periodontal pocket. The specimens were divided into groups for attachment experiment and proliferation experiment. Each included a 24 h group and 48 h group respectively, which was subdivided into subgroups with 18 different conditions, and each condition was carried out in triplicate. The diseased root surfaces were demineralised with EDTA (24%, pH 6.7) for 2 min. Control roots were not exposed to the etching process. All of the specimens were rinsed with phosphate-buffered saline (PBS). Each test slice was autoclaved before treatment with either 20% PRP or 20% PPP. After treating with PRP or PPP, the specimens were sterilised under UV light for 2 h prior to seeding with (3×10^4 cells/ml) HGF, HPDLF or OB, and placed at 37 °C in a humidified atmosphere for 1 h. All test samples were rinsed with PBS to remove excess PRP/PPP and then were replaced with DMEM in which HPDLFs, HGFs or HOBs were seeded at a cell density of 10^4 cells per well. The samples received each treatment in duplicate. Wells were then incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 h. The controls were treated with PBS alone. Double-sided adhesive tape was used to fix the diseased root slices to the bottom of wells in a 48-well tissue culture plate. The cementum surface of the slice was used as substrate for cell attachment and spreading assays.

Cell attachment analyses

The treated and control root slices were then sterilised under UV light for 2 h prior to seeding with 3×10^4 cells/ml of HGF, HPDLF or HOB. Samples were incubated at 37 °C with 5% CO₂–95% air for a 24 h period following rinsing to remove nonadherent cells. All samples were prepared in triplicate. The attached cells on the root

Table 1 Attachment of three kinds of cells to diseased root surface under different treatment (24 h)

	Control	Demineralisation	PRP	PRP + demineralisation	PPP	PPP + demineralisation
HGF	260.42 ± 26.05	321.18 ± 15.03*	355.90 ± 39.78*	494.64 ± 25.82Δ	347.22 ± 39.78*	485.96 ± 29.81Δ
PDLFS	251.74 ± 15.03	312.50 ± 26.04*	347.22 ± 30.07*	485.96 ± 29.81Δ	321.18 ± 39.78*	460.07 ± 30.07Δ
OB	269.10 ± 30.07	329.86 ± 15.03*	381.95 ± 39.78*	511.85 ± 14.77Δ	338.54 ± 26.04*	447.28 ± 39.53Δ

* $P < 0.05$, $\Delta P < 0.01$

specimen were collected by trypsin–EDTA treatment and then cell numbers were counted.

Viability and proliferation assays

Cell viability was assayed using an assay based on the cellular conversion of a tetrazolium salt (MTT) into a blue formazan product that can be read using a computer-assisted ELISA microplate reader. The optical density at 490 nm absorbance is directly proportional to the number of viable cells on the specimen chips. All samples were prepared in triplicate. They were incubated at 37 °C with 5% CO₂–95% air for a 24 h and 48 h period. After incubation, duplicate 100 µl samples were then taken from each well and placed into 96-well plates. The 96-well plates were then placed into an ELISA plate reader and optical densities were obtained using ELX800 devices (BioTek Instruments Inc.).

Scanning electron microscopy evaluation of attachment

Duplicate cultures were used for the scanning electron microscopy evaluation. Following incubation, cell-seeded diseased root slices were rinsed with PBS and fixed for 48 h using 4% paraformaldehyde–1.25% glutaraldehyde in PBS + 4% sucrose, with pH 7.2. The root slices were washed in buffer (PBS + 4% sucrose) and post-fixed in 1% osmium tetroxide (OsO₄) + PBS for 30 min. Samples were dehydrated with three rinses for 15 min in 70%, 90%, 95% and finally 100% ethanol and then carefully removed from the wells and exposed to a critical-point drying process. The samples were individually mounted onto scanning electron microscopy stubs with the cell-seeded surface orientated upwards and coated with a carbon–gold alloy to visualise the attachment and proliferation under a scanning electron microscope (AMRAY 1000B, USA).

Statistical analysis

Data were expressed as the mean plus/minus standard deviation. The results were statistically analysed using

one-way analysis of variance and then compared between two groups. The statistical significance level was set at $P < 0.05$ or $P < 0.01$.

Results

Attachment analysis

Table 1 displays the results of the attachment of cells to untreated or treated diseased root surfaces. When the cells were exposed to untreated diseased root surfaces, the initial attachment of all three cell types showed very few cells attached and spread over the treated root surface. Treatment of the root surfaces with EDTA, PRP and PPP alone all significantly increased cell attachment for all cell types ($P < 0.05$). When PRP or PPP were added to previously EDTA-etched root surfaces, cell attachment was further enhanced ($P < 0.01$ both for PRP and PPP).

Viability and proliferation analysis

Using the vital dye MTT, all cells attached to the root surfaces were confirmed to be vital. This dye could also be used as a means to determine cell numbers and, thus, give a measure of cell proliferation over time. Tables 2 and 3 show the proliferative responses of the cells to the various root surface treatments over a 24–48 h period. The results indicate that diseased root surfaces treated with PRP or PPP alone showed slightly increased cell proliferation compared with the untreated diseased root surfaces, but were not significant statistically ($P > 0.05$). When PRP or PPP was added to previously EDTA-etched root surfaces, cell proliferation was also further enhanced significantly after the 48 h period ($P < 0.05$).

Scanning electron microscopy analysis of attachment

Electron microscopy evaluation of cell attachment to the root surfaces confirmed that both PRP- and PPP-treated diseased root surfaces significantly enhanced cell attachment (Figs 1 to 3). The differences in root surface morphology of healthy, diseased and demineralised diseased

Table 2 Proliferation of three kinds of cells to diseased root surface with PRP treatment (OD Value, 24h-48h)

	Control group		Demineralisation group		PRP		PRP + demineralisation	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
HGF	0.261 ± 0.005	0.216 ± 0.024*	0.297 ± 0.016	0.320 ± 0.012	0.310 ± 0.021	0.355 ± 0.027	0.394 ± 0.010	0.446 ± 0.018*
PDLFs	0.257 ± 0.017	0.215 ± 0.012*	0.295 ± 0.014	0.319 ± 0.013	0.307 ± 0.018	0.324 ± 0.026	0.388 ± 0.017	0.436 ± 0.010*
OB	0.276 ± 0.015	0.228 ± 0.020*	0.321 ± 0.019	0.348 ± 0.021	0.331 ± 0.020	0.360 ± 0.012	0.411 ± 0.010	0.453 ± 0.020*

P* < 0.05Table 3** Proliferation of three kinds of cells to diseased root surface with PPP treatment (OD Value, 24h-48h)

	Control group		Demineralisation group		PPP		PPP + demineralisation	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
HGF	0.261 ± 0.005	0.216 ± 0.024*	0.297 ± 0.016	0.320 ± 0.012	0.302 ± 0.023	0.307 ± 0.021	0.383 ± 0.014	0.436 ± 0.019*
PDLFs	0.257 ± 0.017	0.215 ± 0.012*	0.295 ± 0.014	0.320 ± 0.019	0.305 ± 0.010	0.319 ± 0.017	0.379 ± 0.020	0.422 ± 0.010*
OB	0.276 ± 0.015	0.228 ± 0.020*	0.321 ± 0.019	0.348 ± 0.021	0.331 ± 0.026	0.354 ± 0.014	0.405 ± 0.009	0.443 ± 0.018*

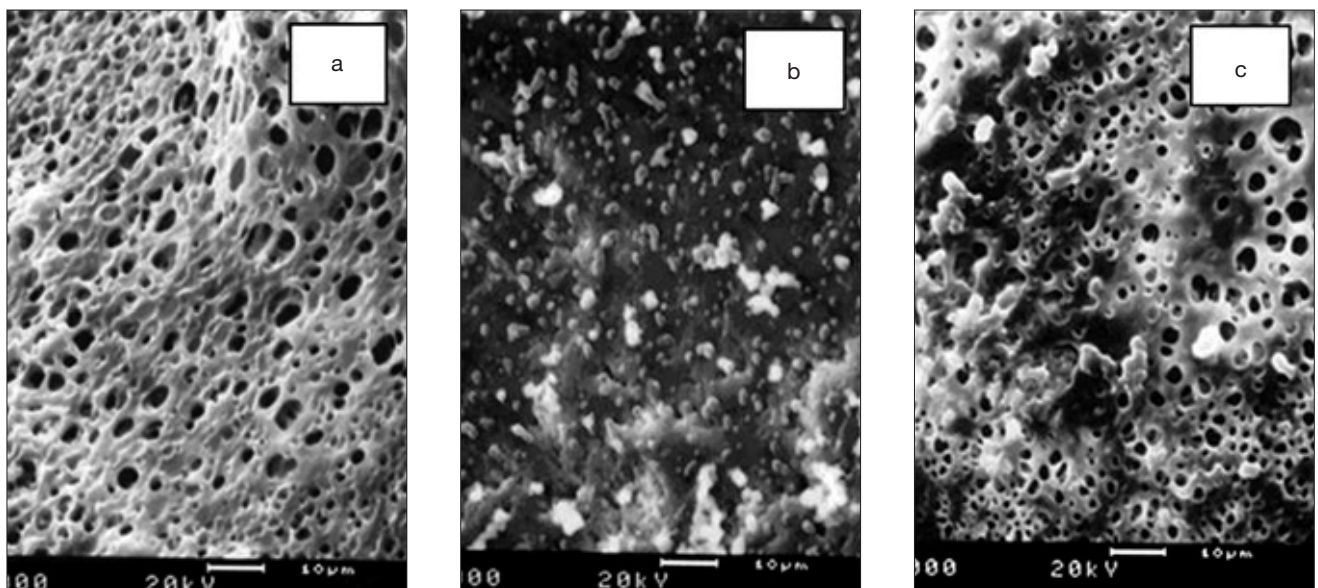
**P* < 0.05

root surfaces are shown in Fig 1. The debris and surface changes were very evident on the diseased root surface compared with the 'cleaner' and porous nature of the healthy and demineralised surfaces. The cells attached to the root surfaces treated with EDTA exhibited numerous filipodia and lamellopodial extensions (Fig 3). While the addition of PRP or PPP to the demineralised surfaces enhanced the number of cells present, their morphology was no different than those cells attached to the etched

surface alone (Figs 2 and 3). These observations were regular and consistent in all specimens examined.

Discussion

Regenerative agents administered directly into periodontal defects should exert little or no damage to the cell necessary for the regenerative process. In order for periodontal regeneration to occur successfully, periodontal

**Fig 1** Scanning electron micrograph of a healthy root surface (a), diseased root surface (b), and diseased root surface demineralised with EDTA (c) (×1000).

fibroblasts need to attach and grow effectively on root surfaces previously exposed to the periodontal pocket environment. Numerous studies have shown that the connective tissue reattachment is biologically possible and that the characteristics of the diseased root surfaces are of great importance in the regeneration of tissue^{18,25-27}. The present study compared, *in vitro*, the initial attachment and proliferation of HGFs, HPDLFs and HOBs on periodontally diseased root surfaces that had been exposed to demineralisation and subsequent coating with PRP or PPP. It was found that the initial attachment of the fibroblasts on the diseased root surfaces was similar. The

poor attachment of cells to untreated diseased root surfaces is in agreement with the results of other studies²⁸⁻³¹. The reduced number of cells attaching and spreading on the diseased root surfaces indicated a poor biocompatibility of these surfaces. Indeed, surface contamination of root surfaces exposed to the periodontal pocket has long been considered to be a limiting factor for cell attachment. In addition, surface changes to the molecular composition of the exposed cementum surface have been reported recently³². Such findings have supported the concept of careful root surface debridement to remove such contaminants and altered composition to

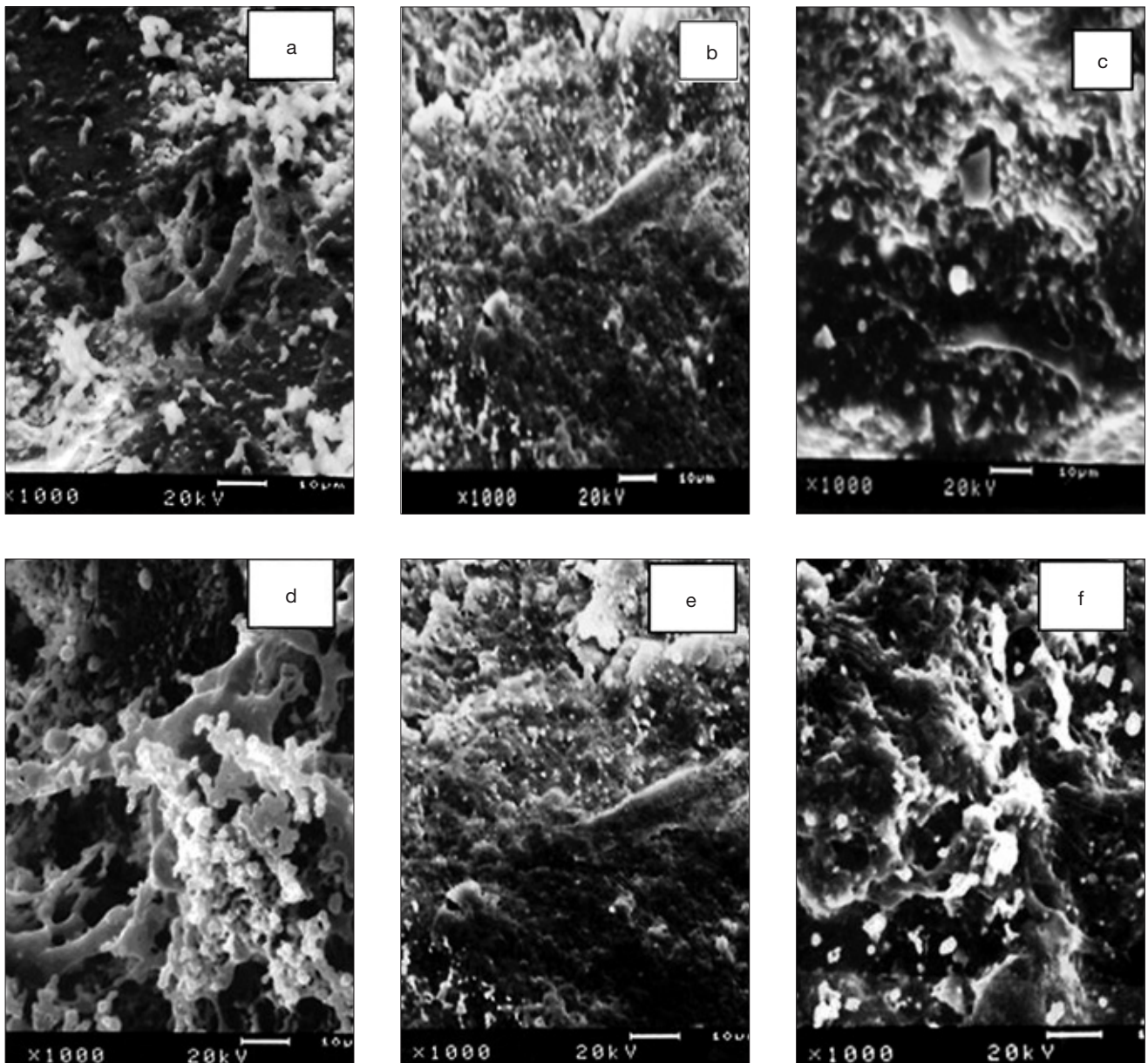


Fig 2 Scanning electron micrograph of HGFs, HPDLFs and HOBs cultured on a diseased root surface (a–c respectively), or on a diseased root surface demineralised with EDTA (d–f respectively) ($\times 1000$).

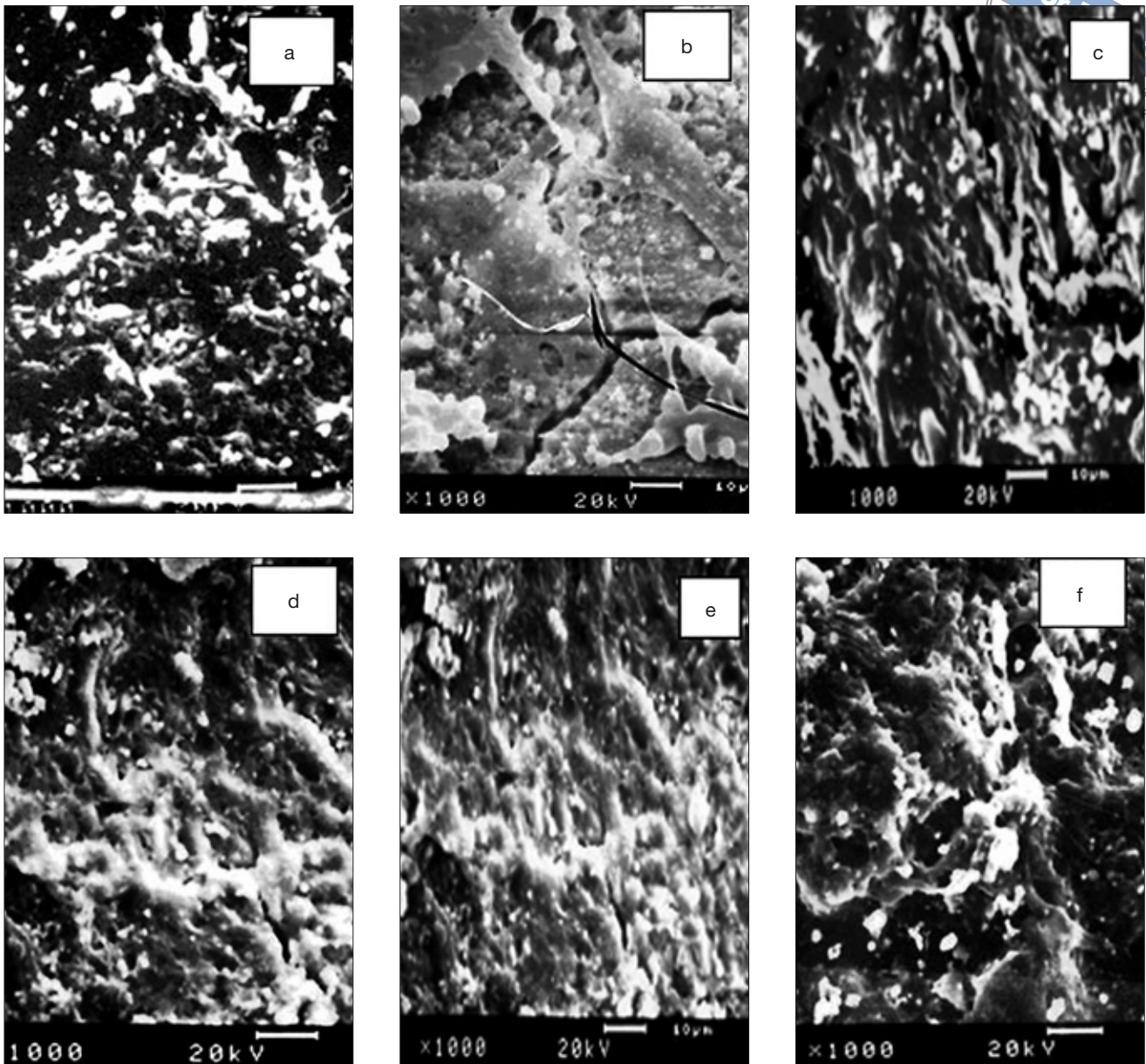


Fig 3 Scanning electron micrograph of HGFs, HPDLFs and HOBs cultured on a diseased root surface treated with PRP (a–c respectively) or PPP (d–f respectively) ($\times 1000$).

make the root surface biocompatible. Despite careful root surface debridement, it is apparent that this is still not sufficient to allow for predictable new cementum formation and attachment of periodontal ligament in the clinical setting. Hence, current research has focused on the coating of prepared root surfaces with biological agents to enhance periodontal repair and regeneration.

Early attempts to improve the biocompatibility of root surfaces included demineralisation with agents such as

citric acid or EDTA³³⁻³⁵. While both agents can render the root surface more conducive to cell attachment, their clinical utility has been limited³⁶⁻³⁸.

PRP contains a large number of growth factors, including platelet-derived growth factor BB, transforming growth factor β , insulin growth factor 1, platelet-derived epidermal growth factor, platelet-derived angiogenesis factor and platelet factor 4, which are very important components in wound repair processes^{14,39}.

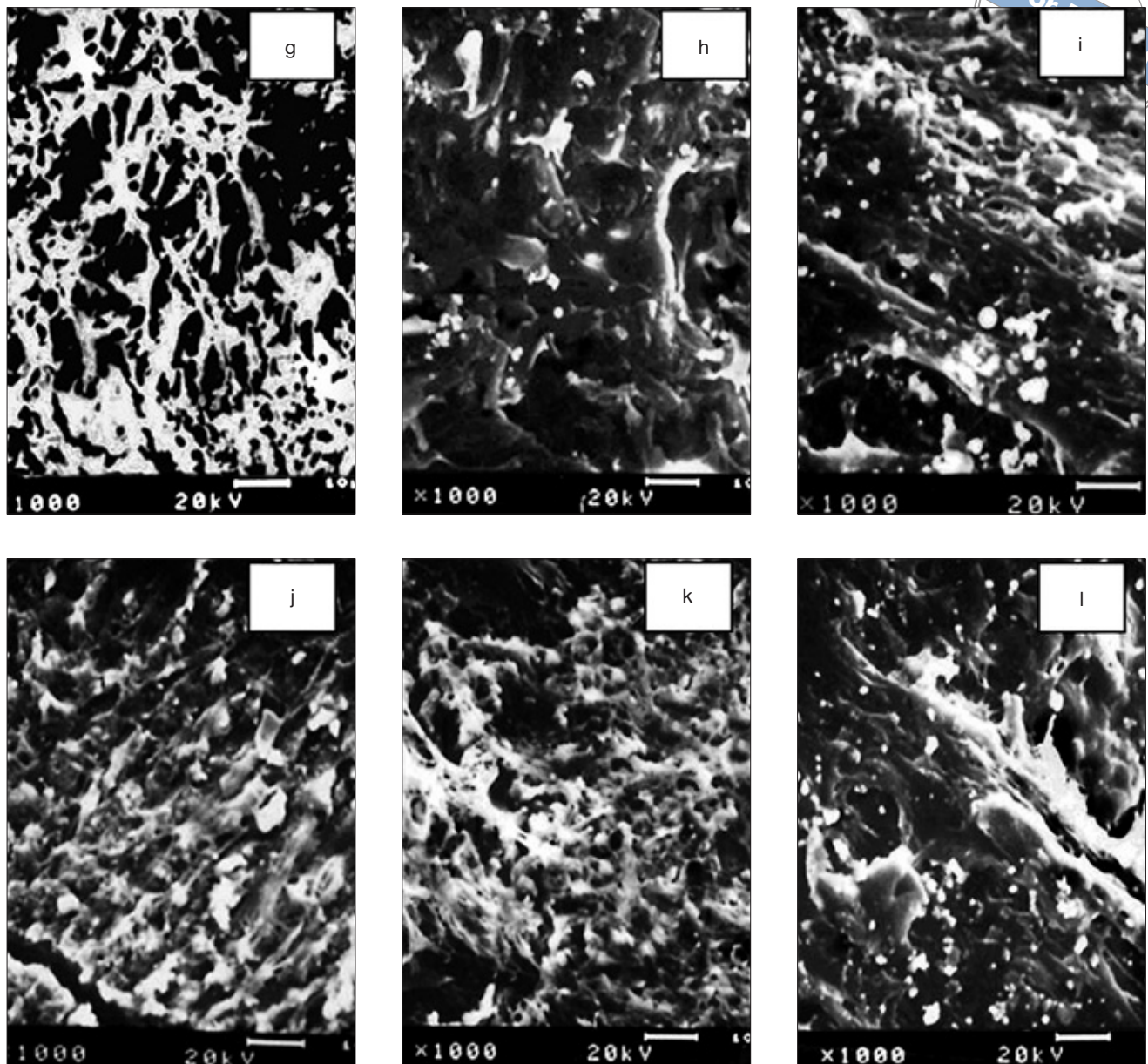


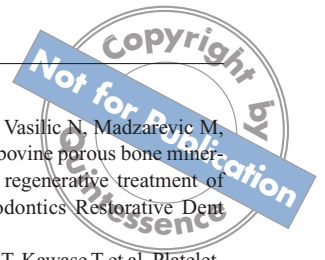
Fig 3 Scanning electron micrograph of HGFs, HPDLFs and HOBs cultured on a diseased root surface demineralised with EDTA and treated with PRP (g–i respectively) or with PPP (j–l respectively) ($\times 1000$).

On the other hand, PPP is rich in adhesion proteins such as fibrin⁴⁰. Both PRP and PPP have been proposed to be of potential use in tissue regeneration^{28,40,41}. Previous studies have confirmed that PRP is capable of aiding periodontal regeneration^{42–47} and modulating the proliferation of periodontally related cells *in vitro*^{14,47}.

The present study has demonstrated that while root surface demineralisation can aid cell attachment, this important biological event can be further enhanced by

the application of either PRP or PPP. These findings are in agreement with an earlier study reporting the enhanced attachment of cells to GTR membranes⁴¹.

Scanning electron microscopy assessment of the morphology of cells attached to treated root surfaces indicated that in the presence of PRP and PPP the cells appeared to be better adapted to their environment and well attached to the underlying substratum. This contrasted with the appearance of the few cells which were



attached to the untreated diseased root surfaces. Significant morphological changes have been reported for cells attached to either periodontal disease-affected roots or root surfaces previously treated with lipopoly-saccharide.

While *in vitro* studies must be interpreted with caution, the results of the present study indicate that root surface treatment with specific biological agents can have a beneficial effect with regard to cell attachment and spreading and that this could be of benefit to enhancing periodontal regeneration. Further work is needed to establish the usefulness of such products.

In conclusion, this study has demonstrated that the addition of either PRP or PPP to previously scaled and chemically demineralised root surfaces can enhance cell attachment and proliferation. It is clear that PRP/PPP has a significant impact on the way these cells attach to the demineralised root surfaces by both increasing the amount and quality of the attachment. However, how this increase in attachment is achieved and how well these *in vitro* results are translated into actual clinical benefits still needs further investigation.

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