

Growth Factors, CD34 Positive Cells, and Fibrin Network Analysis in Concentrated Growth Factors Fraction

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ABSTRACT An interesting clinical option for optimizing healing tissue is the use of platelet concentrate. Platelets contain high quantities of growth factors, among these TGF- β 1 and VEGF, which are known to be implicated in tissue regeneration. CGF is produced by processing blood samples with a special centrifuge device; three layers are formed: top acellular plasma (PPP), middle CGF and bottom red blood cells (RBC) layers. Given that to date there are no data concerning the biological characteristic of CGF, the aim of this study was to evaluate the presence of TGF- β 1 and VEGF in CGF and also in PPP and RBC layers. In addition, since circulating stem cells are recruited from blood to injured tissue for healing we also evaluated the presence of CD34 positive cells. Our data show the presence of TGF- β 1 and VEGF in CGF and RBC layers. In addition, we show CD34 positive cells in CGF. *Microsc. Res. Tech.* 74:772–777, 2011. © 2010 Wiley-Liss, Inc.

INTRODUCTION

The wound healing process is mediated by a wide range of intracellular and extracellular events. An interesting clinical option to optimize the healing of hard and soft tissue is using platelet concentrates.

Platelets contain high quantities of growth factors such as platelet-derived growth factor (PDGF), transforming growth factor- β 1 (TGF- β 1) and β 2 (TGF- β 2), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF), which stimulate cell proliferation, matrix remodeling, and angiogenesis (Intini, 2009).

Recently, numerous techniques using platelet concentrate have been developed to obtain different ratios of platelets, growth factors, leukocytes, and fibrin matrix (Anitua et al., 2008; Dohan et al., 2006a,b; Fernández-Barbero et al., 2006).

The first generation of platelet concentrate is platelet rich plasma-PRP. PRP has been used to accelerate tissue healing for a long time; nevertheless its effects are contradictory inducing cell proliferation/differentiation or even the opposite effects (Dohan Ehrenfest et al., 2009). Choukroun's platelet rich fibrin-PRF is the latest development of these protocols (Choukroun et al., 2006; Dohan Ehrenfest et al., 2009; Simonpieri et al., 2009; Sunitha Raja et al., 2008). A patient's venous blood sample is taken without anticoagulant (Dohan Ehrenfest et al., 2009; Gassling et al., 2009; He et al., 2009; Su et al., 2009). After centrifugation, three layers are formed in the tube: red blood cells at the bottom, acellular plasma on the top, and a clot of PRF between them. Unlike most platelet concentrates, which show a fibrin glue-like consistency and quickly dissolve when applied, PRF have a solid fibrin consistency and does not dissolve quickly, providing a matrix that contains high

concentration of platelets, leukocytes and growth factors (Gruber et al., 2004; Simonpieri et al., 2009; Weibrich et al., 2005).

Concentrated growth factors (CGF) was developed by Sacco in 2006. CGF is produced by centrifuging blood samples with a special centrifuge device (Medifuge, Silfradent srl, Italy), similar to PRF. Nevertheless, the different centrifugation speed permits the isolation of a much larger, denser and richer in growth factors fibrin matrix.

PRF has been used in different clinical applications as well as engineering tissue (Coetzee et al., 2005; Dohan Ehrenfest et al., 2009; Rutkowski et al., 2008), nevertheless CGF seems to have a better regenerative capacity and versatility, as reported for sinus and alveolar ridge augmentation (Sohn et al., 2009).

The potential of these preparations is the fibrin network that contains platelets, leukocytes, and growth factors and provides a matrix for cell migration, i.e., fibroblasts and endothelial cells involved in angiogenesis and tissue remodeling (Gassling et al., 2009). In particular, platelets are important since they release high concentrations of biologically active proteins and support recruitment, growth and cell morphogenesis (Anitua et al., 2009; He et al., 2009; Nurden et al., 2008). On the contrary, the role of leukocytes is presently debated: some authors suggested that leukocytes should be discarded to avoid the inflammatory process (Anitua et al., 2008), others underline their importance

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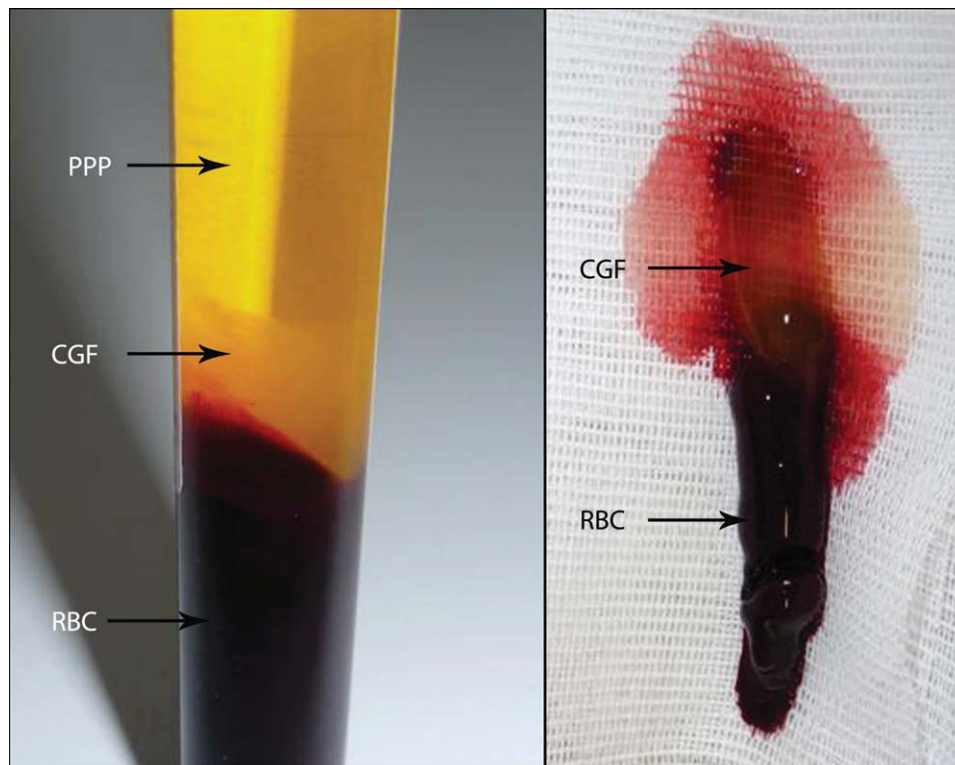


Fig. 1. Blood sample after centrifugation without anticoagulant. Three layers are obtained: PPP, upper layer; CGF, middle layer; RBC, lower layer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

as regulators of slow growth factor release by PRF (Intini, 2009; Rutkowski et al., 2008).

Given that to date, there is very little information about CGF, the principal aim of this study is to evaluate the presence of some important growth factors (TGF- β 1 and VEGF) involved in tissue regeneration; moreover, we quantify the TGF- β 1 and VEGF levels also in PPP and RBC layers.

Finally, since circulating stem cells are recruited from blood to injured tissue by platelets (Greiling and Clark, 1997; Gruber et al., 2004), we evaluate the presence of CD34 positive cells (Asahara et al., 1997).

MATERIALS AND METHODS

Blood Sample Centrifugation

Venous blood samples were collected from six healthy volunteers, three males and three females, nonsmokers, aged between 30 and 47 years old.

The CGF was produced as follow: 9 mL of blood was drawn in sterile Vacuette tubes (Greiner Bio-One, GmbH, Kremsmunster, Austria) without anticoagulant solutions. These tubes were then immediately centrifuged in special machine (Medifuge MF200, Silfradent srl, Forlì, Italy) using a program with the following characteristics: 30" acceleration, 2' 2,700 rpm, 4' 2,400 rpm, 4' 2,700 rpm, 3' 3,000 rpm, and 36" deceleration and stop. At the end of the process there are three blood fractions: (1) the upper platelet poor plasma (PPP) layer; (2) the middle fibrin rich gel with aggre-

gated platelets and concentrated growth factors (CGF); (3) the lower red blood cell (RBC) layer (Fig. 1).

Histological and Immunohistochemical Analyses

CGF and RBC layers were fixed in 10% neutral buffered formalin solution pH 7.2 for 48 h and embedded in paraffin according to standard procedure. Twenty serial sections (7- μ m thick) of each sample were cut using a microtome and then they used for the histological and immunohistochemical analyses.

Alternate sections were processed for Hematoxylin-Eosin and Giemsa staining according to standard procedures and then examined using a light microscope (Olympus, Germany) at a final magnification of 100 \times .

For immunohistochemical analysis the sections were deparaffinised in xylene, rehydrated in descending concentrations of ethanol solutions, water, and phosphate buffer saline (PBS) and subjected to antigen retrieval in 0.05 M sodium citrate buffer (pH 6.0) in a microwave oven for two cycles of 5 min at 650 W and one cycle of 3 min at 400 W (Rodella et al., 2006). Endogenous peroxidase activity was blocked by incubation with a solution of 3% hydrogen peroxide in methanol for 30 min. Sections were then incubated with appropriate normal serum (Vector Labs., Burlingame, CA) for 1 h, and treated for 1 h at room temperature and overnight at 4 $^{\circ}$ C with mouse monoclonal antibody against TGF- β 1 (diluted 1:50, TGF- β 1(TB21):sc-52893, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody against VEGF (diluted 1:50, VEGF(147):

sc-507, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal antibody against CD34 (diluted 1:30, Thermo Scientific, Fremont, CA). Then, the sections were sequentially incubated with appropriate biotinylated secondary antibodies, avidin-biotin peroxidase complex (Vector Labs, Burlingame, CA) and, finally, in a solution of 0.05% 3-3'-diaminobenzidine tetrahydrochloride (Sigma Aldrich, St. Louis, MO) and 0.33% hydrogen peroxide. All sections were counterstained with hematoxylin, dehydrated, and mounted. Control reactions were performed by omitting the primary antibody and with isotype-matched irrelevant IgGs as negative control.

Quantitative Analysis of TGF- β 1, VEGF, and CD34 Expression

The immunohistochemical data of TGF- β 1, VEGF, and CD34 expression were evaluated quantitatively. For the TGF- β 1 and VEGF quantitative analysis, immunostaining intensity was evaluated blindly, using an optical light microscope (Olympus, Germany) at a final magnification of 100 \times . Digitally fixed images of slices were analyzed using an image analyzer (Image Pro-Plus, Milan, Italy) and were calculated as integrated optical density (IOD) in five random fields with the same arbitrary area for each section. For the CD34 quantitative analysis, the total number of CD34 positive cells in 10 high-power fields (hpf) with the same arbitrary area for each section was evaluated blindly, at a final magnification of 40 \times , as previous described by Elliot et al. (2005) ten section for subject were analyzed.

The data were pooled to give a mean value \pm SD and a statistical analysis was applied to compare the results obtained from the different samples.

Scanning Electron Microscopy (SEM) Analysis

CGF layer samples were fixed in 2% glutaraldehyde for 1 h, were rinsed in cacodylate buffer solution and fixed for 1 h with 1% osmium tetroxide (OsO₄). Then the samples were dehydrated serially in 30, 50, 70, 90, and 100% ethanol solutions. The SEM procedures were completed by critical drying point of the material. Finally the CGF samples were observed at 20 kV using Sem LEO 1430 scanning electron microscope.

Immunoblotting Analysis for TGF- β 1 and VEGF Proteins

For immunoblotting analysis, the CGF and the RBC layer samples were homogenized and centrifuged at 12,000 rpm for 2 min at 4 $^{\circ}$ C. Protein concentration was assessed using Albumin Standards (Sigma Aldrich, St. Louis, US) according to the manufacturer's instruction. The samples (100 μ g μ L⁻¹) were analyzed by 10% SDS-PAGE and electro-transferred to a nitro-cellulose membrane (pore size 0.45 μ m; Bio-Rad, Bio-Rad Laboratories srl, Milan, Italy) by wet blotting (350 mA for 1 h, 4 $^{\circ}$ C). The membrane was blocked with 5% bovine serum albumine (BSA, Sigma Aldrich, St. Louis, US) in Tris-buffered saline Tween-20 (TTBS) at 4 $^{\circ}$ C overnight. After washing with TTBS, proteins were incubated 1 h at room temperature in mouse monoclonal antibody against TGF- β 1 [diluted 1:200, TGF- β 1(TB21):sc-52893, Santa Cruz Biotechnology, Santa Cruz, CA] and rabbit polyclonal antibody against VEGF [diluted

1:1000, VEGF(147): sc-507, Santa Cruz Biotechnology, Santa Cruz, CA], place in a solution of appropriate biotinylated secondary antibodies (Vector Labs, Burlingame, CA), avidin-biotin-peroxidase complex (ABC kit, Vector Labs, Burlingame, CA) and, finally, in a solution of 0.05% 3-3'-diaminobenzidine tetrahydrochloride (Sigma Aldrich, St. Louis, US) and 0.03% hydrogen peroxide.

ELISA Analysis

TGF- β 1 and VEGF-A concentration in the PPP layer of all samples were quantified using a commercially available ELISA (enzyme-linked immunosorbent assay) kit (Bender MedSystems GmbH, Vienna, Austria) following the manufacturer's instruction. The absorbance was read using a microplate reader ELISA at 450 nm and then the concentrations were calculated.

Statistical Analysis

The results were expressed as mean \pm standard deviation (SD). All data were analyzed by ANOVA test corrected by Bonferroni. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Scanning Electron Microscopy (SEM) Analysis

Clear images of the elements that constituted CGF fractions were obtained by SEM analysis. In particular, we observed a fibrin network constituted by thin and thick fibrillar elements (Fig. 2A). Multiple platelet cell elements were observed forming a cell aggregate trapped among the fibrin network (Fig. 2B).

Growth Factors (TGF- β 1 and VEGF) in the Different Blood Fractions

Immunohistochemical evaluation of TGF- β 1 and VEGF expression in the CGF and RBC layers showed a widespread immunostaining in both fractions (Figs. 3A–3D). The quantitative analyses by IOD measurements showed a similar level between the CGF and the RBC layers both for TGF- β 1 (30.39 \pm 2.92 vs. 30.24 \pm 2.27) and VEGF (28.96 \pm 1.97 vs. 27.14 \pm 0.52), as reported in Figure 3E. Moreover, the presence of TGF- β 1 (12.5 kDa) and VEGF (15 kDa) were confirmed by immunoblotting analysis (Fig. 3F).

Considering the low levels of growth factors in the PPP layer, the quantification of the TGF- β 1 and VEGF was made using ELISA kit assay. The results obtained showed a small amount of both growth factors (TGF- β 1 18,950 \pm 7,500 and VEGF 335.15 \pm 38.11, Table 1).

Quantitative Analysis of CD34 Positive Cells

CD34 positive cells were evaluated using immunohistochemical method. The results showed the presence of CD34 positive cells both in the CGF and RBC layers (Figs. 4A and 4B). Nevertheless, the quantitative analysis, which statistically compared the number of CD34 positive cells in 10 hpf for ten sections, showed a significantly higher number of CD34 positive cells in the CGF (153.7 \pm 15.04) with respect to the RBC (14.3 \pm 1.61) layer (Fig. 4C).

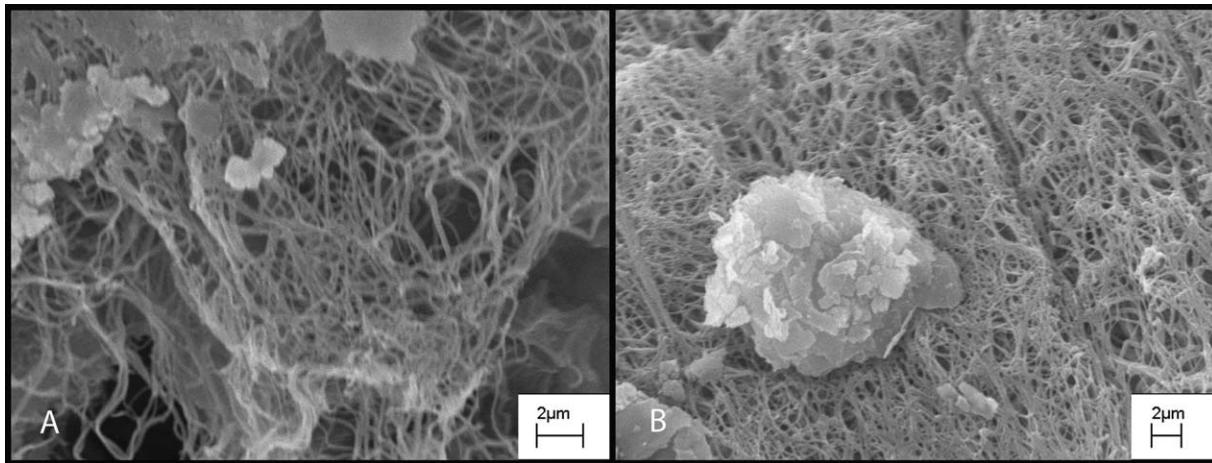


Fig. 2. SEM analysis of CGF. Fibrin network (A) in which are trapped multiple platelet cell elements (B).

TABLE 1. Quantity of released TGF- β 1 and VEGF in PPP layer

	TGF- β 1 (pg mL ⁻¹)	VEGF (pg mL ⁻¹)
PPP	18,950 \pm 7,500	335.15 \pm 38.11

DISCUSSION

CGF is an innovative method for producing a fibrin matrix with concentrated growth factors and its clinical applications are presently being investigated.

Compared to PRF, which has been widely described (Dohan et al., 2006a,b; Dohan Ehrenfest et al., 2009; Fernández-Barbero et al., 2006; Gürbüz et al., 2010), very little data concerning the characteristics and the use of CGF are available. In particular, CGF was reported to have a good regenerative capacity and a high versatility on sinus and alveolar ridge augmentation (Sohn et al., 2009).

Since there are no data concerning CGF biological characteristics, our aim was first of all, to evaluate the morphological ultra-structure and to assay the presence of some growth factors (TGF- β 1 and VEGF), which are known to be implicated in tissue regeneration.

Similarly to PRF (Dohan Ehrenfest et al., 2009, 2010; Dohan et al., 2006a,b; Mosesson, 2005), CGF has a complex tridimensional architecture which makes it a real platelet, leukocyte and growth factor-rich fibrin biomaterial. In particular, the platelets and the dense fibrin network make large clusters of coagulation in the initial millimetres of the membrane beyond the red blood cell base, as confirmed by SEM analysis.

According to the literature, the presence of growth factors, such as TGF- β 1 and VEGF, is important for stimulating cell proliferation, matrix remodeling, and angiogenesis during healing processes (Grainger et al., 2000; Intini, 2009; Rodella et al., 2010). Previous studies investigated the presence of many different residual cytokines (growth factors and interleukins) within the PPP fraction and PRF network (Dohan Ehrenfest et al., 2009; Gürbüz et al., 2010; He et al., 2009; Su et al., 2009), reporting very low levels in PPP compar-

ing with the PRF layer (Dohan Ehrenfest et al., 2009). Our data confirmed the presence of TGF- β 1 and VEGF in CGF and demonstrate a similar pattern of expression also in RBC layer.

These results support the clinical data, confirming that growth factors are important in tissue regeneration; nevertheless, the presence of growth factors in RBC layer has to be considered.

We know that platelets are not the only blood cells which contain growth factors but also leukocytes and erythrocytes also contain TGF- β 1 (Grainger et al., 2000) and VEGF factors (Kusumanto et al., 2003; van der Loos et al., 2010). Therefore, if we can say that improved CGF procedure could optimize the amount of growth factors in CGF layer decreasing their content in RBC layer, a possible use of RBC layer in clinical application could also be considered.

Another aim of this study was to quantify, by immunohistochemical methods, the number of CD34 positive cells in CGF and RBC layers. It is known that PRF could provide a supportive matrix for circulating mesenchymal stem cells (Agis et al., 2010; Gassling et al., 2009), which are recruited from blood to injured tissue by signalling molecules released from platelets (Greiling and Clark, 1997; Gruber et al., 2004). Moreover, increasing evidence points to the role of circulating CD34 positive cells (Asahara et al., 1997; Kikuchi-Taura et al., 2006; Matic et al., 1997) in vascular maintenance, neovascularisation, and angiogenesis (Ademokun et al., 1997; Majka et al., 2001). Our results show CD34 positive cells in both layers, recording a higher number in CGF. This could be explained by CGF network composition in which the cells are trapped. Since, to date, there are no literature data regarding the implications of CD34 positive cells present in CGF in clinical applications, further studies should be focused on this topic.

In conclusion, our study demonstrates the presence of TGF- β 1 and VEGF in CGF and RBC layers, suggesting that improved CGF procedure could optimize the amount of growth factors in the CGF layer or, alternatively, a possible use of RBC layer in clinical applications. Moreover, the presence of CD34 positive cells

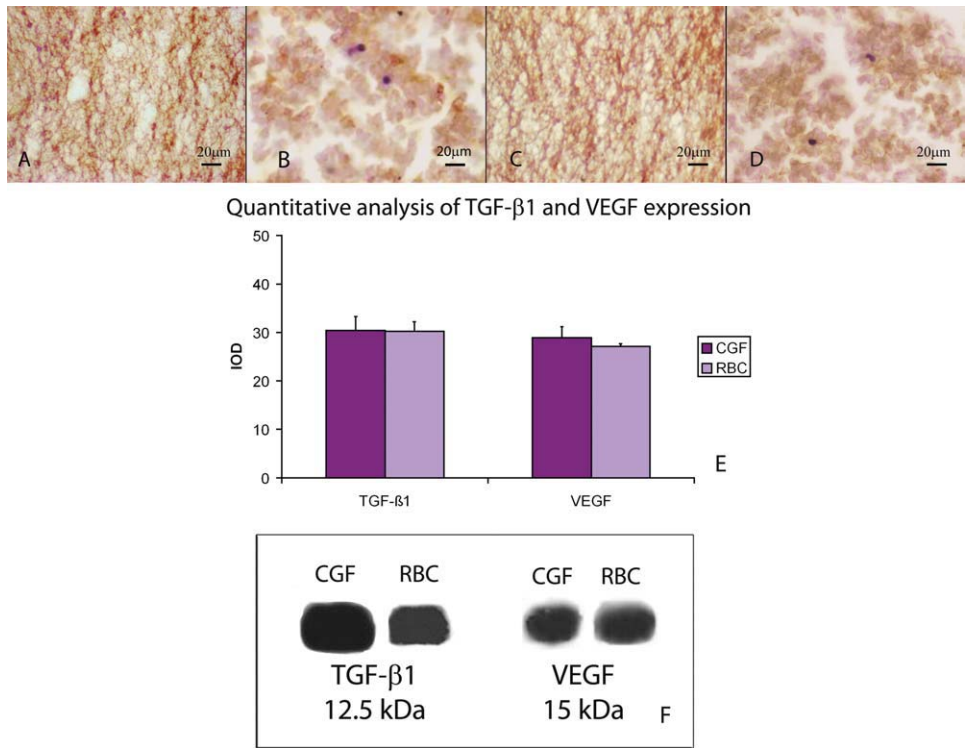


Fig. 3. Immunohistochemical staining of TGF-β1 in CGF (A) and RBC (B) layers; immunohistochemical staining of VEGF in CGF (C) and RBC (D) layers. The graph showed the quantitative analysis (IOD) of TGF-β1 and VEGF expression in CGF

and in RBC layers (E). Immunoblotting analysis of TGF-β1 and VEGF in CGF and RBC layers (F). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

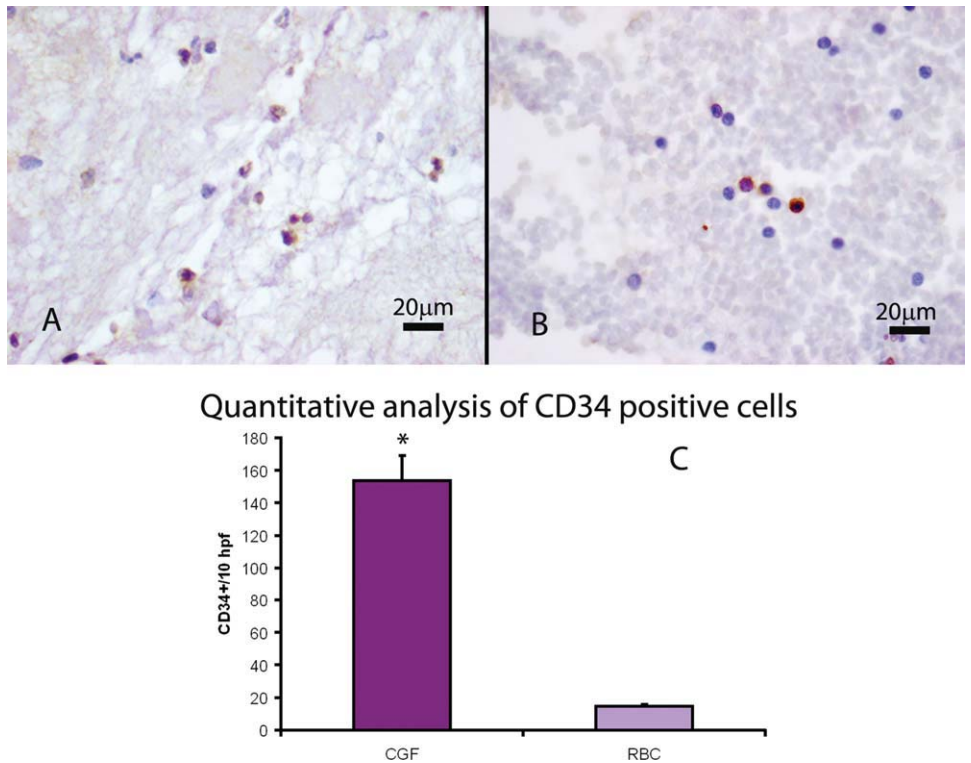


Fig. 4. Immunohistochemical staining of CD34 in CGF (A) and RBC (B) layers. The graphs showed the number of CD34 positive cells in 10 hpf for ten sections in CGF and in RBC layers (C). **P* < 0.05 versus RBC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

within the CGF network could lead to investigate their clinical implications.

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