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Inflammatory infiltrate, microvessel density, Vascular Endothelial Growth Factor (VEGF), Nitric Oxide Synthase (NOS), and proliferative activity in soft tissues below intraorally welded titanium bars

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Abstract

Background: The aim of the present study was a comparative evaluation of inflammatory infiltrate, microvessel density (MVD), Vascular Endothelial Growth Factor (VEGF), Nitric Oxide Synthase (NOS), and proliferative activity in soft tissues below intra-orally welded titanium bars. **Materials and Methods:** Twenty-two patients participated in this study. All patients carried immediately loaded one stage titanium implants splinted with intra-orally welded titanium bars. Each patient underwent two gingival biopsies: a control biopsy harvested from an area of mucosa 5 mm away from the titanium bar, and a test biopsy from the mucosa below the titanium bar, which were histologically and immunohistochemically processed. **Results:** No fractures or radiographically detectable alterations of the welded frameworks were present. In all the cases examined, the average of the mPI was 1, no suppuration, or BOP was present; probing depth was ≤3 mm. However, the immunohistochemical analysis revealed some differences. The inflammatory infiltrate was mostly present in test sites and its extension was much larger than in control sites. Statistically significant differences were found in MVD and Ki-67 expression between control and test groups (P <0.0001). The high intensity of VEFG, NOS1, NOS3 expression were mainly detected in test group, whereas the low intensities were mostly expressed in controls, with statistically significant differences (P <0.0001). **Conclusions:** In the present study, the immunohistochemical analysis showed that the tissues below the titanium bars underwent a higher rate of inflammatory and reparative processes. However, further long-term studies, where clinical and immunohistochemical data are collected in parallel, should be conducted for a better understanding of the expression pattern of inflammation markers.

Key-words: immediate loading, immunohistochemistry, intra-oral welding, titanium bar.

The treatment of edentulism with dental implants is a reliable and well accepted solution by patients. The high success rate of dental implants is well documented in the literature; however, some failures are reported.[1-4] The absence of primary stability at the time of surgery is one of the reasons for implants failures.[5] Indeed, the peri-implant bone adjusts its architecture according to its capacity to withstand functional loading; the strains induced by these loads affect the bone remodelling process.[6] If stability is not achieved over the long-term, the microstrain could cause peri-implant bone resorption and implant failure.[7]

A system of extrinsic stabilization of the implant to increase its stability, is desirable. The methods to obtain implants stabilization are: metallic and resin splinting, bar-retained overdentures, provisional framework. Among those techniques, the use of titanium bars intra-orally welded to implant abutments seems to have a significant impact on the peri-implant hard tissues response since it is able to reduce the mechanical stress exerted on each implant.[8,9] Moreover, this technique is feasible with the immediate loading technique. Indeed, several reports have shown the high predictability and advantages of rigid splinting of immediately loaded implants.[10,11]

One of the criticisms brought to the electro-welding method is that the presence of titanium bar causes the occurrence of undercut areas difficult to cleanse, with the presence of bacterial plaque accumulation[12]. This is due to the fact that for technical and aesthetic reasons titanium bars should be positioned close to the gingival.[12] A correlation between plaque accumulation and progressive bone loss around implants has been reported in experimental and clinical studies.[13,14] Plaque accumulation on implant surfaces or abutments induces an inflammatory reaction within the gingival and alveolar mucosa in the same manner as around teeth.[14]

However, to our best knowledge, no studies have assessed plaque accumulation in patients wearing implants welded with titanium bars. Specifically, the biological response of soft tissues to plaque deposits below titanium bars has not been investigated yet.

Indeed, during inflammation the formation of new capillaries occurs; this is due to the budding of endothelial cells, a process called angiogenesis.[15-17] In periodontal tissues as well as peri-implant tissues, angiogenesis seems to be important for the maintenance of tissue health.[15-17] Inflamed tissues seem to enhance the expression of inflammatory mediators, which in turn may promote angiogenesis.[15] Vascular endothelial growth factor (VEGF) has been used for the evaluation of angiogenesis in peri-implant tissues.[18] Further factors of critical importance in the inflammation process are nitric oxide synthase (NOS) and Ki-67. Nitric oxide (NO) is a free radical, produced from L-arginine via isoenzymes named NOS.[19] NO is essential for a vast spectrum of intracellular and extracellular events in a wide variety of tissues,[20] moreover it is important in host defense and homeostasis. It is also regarded as harmful and has been implicated in the pathogenesis of a wide variety of inflammatory and autoimmune diseases.[21,22] Three different isoform of NOS are known; the endothelial NOS (eNOS or NOS1) and the neuronal NOS (nNOS or NOS3) (19,23-27).[23-27] The third form is an inducible isoenzyme (iNOS or NOS2).[24] Ki-67 antigen specific antibody is used in probes to detect cells undergoing division, which is a typical part of inflammation and reparation processes. Ki-67 antigen expression has been detected in the nuclei of proliferating cells in the G1, S, G2, and M phases of the cell cycle, but is absent in quiescent cells (G0 phase).[28]

The aim of the present study was an evaluation of the inflammatory infiltrate, microvessel density, VEGF, NOS, and proliferative activity in soft tissues below intra-orally welded titanium bars in order to investigate the suitability of such treatment for the biological health of soft tissues.

Materials and Methods

Study design

Twenty-two out of forty-two patients, 7 men (31.81%) and 15 women (68.18%) (age ranging from 37 to 70 years, mean age 62 years) were selected for this study from May 1984 to January 2009. Informed written consent was obtained from the patients and the protocol of the study was approved by the Ethics Committee of the University of Chieti-Pescara.

The inclusion criteria were: partial or total edentulism, no need of bone augmentation procedures prior to implant placement, no active infection in the sites intended for implant placement, controlled oral hygiene,[29] good general health with no contributory past medical history. The exclusion criteria were: severe illness, head and neck radiation therapy, chemotherapy, uncontrolled diabetes, uncontrolled periodontal disease, smoking more than 20 cigarettes a day, immunosuppressed patients, severe hypertension, and pregnancy.

All patients were treated (range 2 months to 25 years; mean 26 months) with a total of 195 immediately loaded titanium one stage implants† and the intra-oral bars were all installed at the same time for all the patients, after the post-surgical phase.

Intraoral Welding

The technique used in the present study is a refinement of the technique reported by Mondani and Mondani,[30] and Hruska.[31] The welding process is subdivided into three stages: preparation, welding, and cooling.

In the preparation stage, the two electrodes of the welding pincers were placed on either side of the bar and the abutment, both of which had to be clean and free of any surface oxidation. The copper electrodes were gently put in contact with the bar and the abutment and firm pressure was applied until a perfect joint between the parts to be welded was achieved.

At the welding stage, the copper electrodes of the welding pincers were supplied by an electrical current, which raised the temperature of the two titanium components to a fusion point (nearly

1,660°C). The process took only 2 to 5 milliseconds and ends out with a barely audible clicking sound.

During the cooling stage the copper electrodes dissipated all the heat that was generated without producing any discomfort to the patient or damage to the surrounding tissue, and transmitting any heat to the peri-implant tissues.

Finally, a provisional functional prosthesis was delivered and after three weeks it was replaced by a metallo-ceramic prosthesis (Fig.1a-d). In order to evaluate the clinical conditions of the tissues, a single calibrated periodontist (SF) measured probing depth, modified plaque index (mPI),[32] and bleeding on probing (BOP) in the sites where the implants were placed. Gingiva was considered clinically healthy when the probing depth was ≤3 mm and there was no presence of bleeding upon probing. During follow-ups, clinical observations, such as probing and biofilm accumulation and peri-apical x-rays were performed at every 6 months up to the second year, and then each year.

Immunohistochemical processing

Each patient underwent 2 gingival biopsies at different time points after bar installation a control biopsy taken from an area of mucosa 5 mm away from the distal end of the titanium bar, and test biopsy from the mucosa below the titanium bar in the edentulous area equidistant from two implants. A scalpel (No. 11 blade and / or Beaver No 6900) was used to harvest the samples. The dimensions of the gingival biopsies ranged from 2 × 3 mm to a maximum of 3 × 5 mm.

All specimens were immediately fixed in 10% neutral buffered formalin and accordingly embedded in paraffin. Three micron sections were subsequently obtained with a Leitz 1512 microtome and stained with Hematoxylin-Eosin. The immunohistochemical staining of CD3, CD20, CD68, Factor VIII, VEGF, NOS1, NOS3, and Ki-67 was performed using the strep-ABC (Streptavidine-Biotine-Peroxidase) method. Three micron sections were cut and mounted on poly-L-lysine-coated slides. Paraffin sections were dewaxed by xylene, rehydrated and finally washed in PBS (pH 7.4) for 10 minutes. In order to unmask the antigens, a microwave oven and a 2.1% content of citric acid was used related to the antibodies CD3 (1:10), CD20 (1:50), CD68 (1:50), Factor VIII (1:50), VEGF (1:100), NOS1 (1:150), NOS3 (1:100), and Ki-67 (1:15). The subsequent steps were optimized by automatic staining.‡ Sections were incubated with primary antibody for 30 minutes at room temperature. Slides were rinsed in buffer, and immunoreaction was completed with the Strep-ABC-Peroxidase method, applying the "Peroxidase Detection System" kit by Novocastra§ and utilizing a multi-link as a secondary biotinylated antibody. After incubation with a chromogen employing "liquid DAB substrate pack",** the specimens were counterstained with Mayer's hematoxyline and coverslipped. The amount of the inflammatory infiltrate were evaluated using a semi-quantitative analysis: *low* = +; *intermediate* = ++; *high* = +++. The value was considered *low* (+) in case less than 10%, *intermediate* (++) in case 10% to 50%; and *high* (+++) in case more than 50% of the cells were positive for the investigated factors. The extension of the inflammatory infiltrate was evaluated using a semi-quantitative analysis: each specimen was divided into 3 areas: the 1/3, 2/3, 3/3 (Fig. 2).[33] The evaluations were performed in 10 randomly selected 20× fields for each area.

The CD3, CD20, CD68, Factor VIII, VEGF, NOS1, NOS3 and Ki-67 were evaluated using a quantitative method. A light microscope†† connected to a high resolution video camera and interfaced to a monitor and personal computer was used. This optical system was associated with a digitizing pad and a histometry software package with image-capturing capabilities.‡‡ Ten random fields were chosen for each specimen and percentage of CD3, CD20, CD68, Ki-67 were calculated. Regarding VEGF, NOS1 and NOS3 the evaluation was conducted after having distinguished two different intensities of the expression of *low* and *high*; the intensities were recognized by the PC software as green and red, respectively.

For the MVD count, the antibody against the human factor VIII-related antigen was used to highlight the blood microvessels. All the morphologic structures with a lumen surrounded by factor VIII-positive endothelial cells were considered blood microvessels. A 20× magnification was used, and the individual microvessels profiles were circled to prevent the duplicates or omit counting. For each case, 10 randomly selected fields, corresponding to 1.1 $mm,^2$ were measured. The values were expressed as number of microvessels per square millimeter of gingival tissues (MVD).

All the immunohistochemical measurements were performed by a pathologist (LA), in a blind manner.

Statistical Analysis

Results were statistically analyzed using Wilcoxon matched-pairs signed-ranks test, and statistically significant differences were accepted as $P < 0.05$. The percentages were expressed mean ± standard deviations.

Results

Clinical Observations

No visible fractures or radiographically detectable alterations of the welded frameworks were evident. In all the cases examined, the average of the mPI was 1 (plaque only recognized by running a probe across the smooth marginal surface of the implant), no suppuration, or BOP was present; probing depth was ≤3 mm.

However, the immunohistochemical analysis revealed some differences between control and test groups.

Inflammatory Infiltrate

The inflammatory infiltrate mainly consisted of lymphocytes, plasma cells, and histiocytes. The semi-quantitative analysis showed that in the control group the inflammatory infiltrate was evaluated as + in12, as ++ in 6 and +++ in 4 samples; while in the test group the values were higher: in 9 samples the inflammatory infiltrate was evaluated as + only in 3 samples, ++ in 10 and as +++ in 9. Regarding the extension, in the control group the inflammatory infiltrate penetrated into 1/3 of the mucosa in 11 cases, into 2/3 in 8 and into 3/3 in 3. On the contrary, test samples showed an extension of inflammatory infiltrate for 1/3 in 7 samples, for 2/3 in 8 and for 3/3 in 7 (Fig. 3a-b).

The percentage of CD 3 + cells (T), CD 20 + cells (B) and CD68 + cells (macrophages/histiocytes) was higher in test group than control, even if the difference between groups was not statistically significant (Table 1).

In 1 specimen of the control group, the inflammatory infiltrate ulcerated the mucosa, while in the test group 4 specimens showed mucosa ulceration.

Microvessel Density (MVD)

In the control group only few microvessels were present in the submucosa. In the test group several microvessels were present in the submucosa (Fig. 4a-b), mostly located in areas showing a wider inflammatory infiltrate. The mean number of vessel/mm^2 was statistically higher ($P < 0.0001$) in tests (28.18 ± 3.44) than controls (15.22 ± 4.45) (Table 1).

VEGF

The intensity of VEGF expression was different between the two examined groups. VEGF was positive in endothelium cells, stromal cells and inflammatory cells; however, the evaluation was performed at the level of the endothelium vascular cells. In the test group cells presented a

prevalently high intensity of VEGF expression were prevalent (55.75 ± 4.22) with statistically significant differences with control group (37.52 ± 2.15). On the contrary, in the control group cells mainly showed low intensity of VEGF expression (62.47 ± 2.15) with statistically significant differences (P <0.0001) with test group (44.25 ± 4.22) (Fig. 5a-d) (Table 1).

NOS1

In both groups NOS1 was expressed in all the examined tissues, even though the intensity of expression was different between the two groups. The evaluation was performed at the level of the endothelium vascular cells. The test group presented higher percentages of high intensity of expression (55.09 ± 4.83) than control group (35.59 ± 7.36), with statistically significant differences (P <0.0001); while, control presented a higher percentages of low intensity of expression (62.63 ± 2.41) than test group (44.90 ± 4.83) (Fig. 6a-d). This difference was statistically significant (P <0.0001) (Table 1).

NOS3

NOS3 was expressed in all the examined tissues of both groups. The intensity of the expression, however, was different between the two groups. The evaluation was performed at the level of the endothelium vascular cells. It was observed that the cells of group test presented higher percentages of high intensity of expression (54.92 ± 4.56) than the cells of control group (37.62 ± 2.82), with statistically significant differences (P <0.0001). The control group cells presented statistically higher percentages (P <0.0001) of low intensity of expression (60.96 ± 5.37) than group test cells (45.07 ± 4.56) (Fig. 7a-d) (Table 1).

Proliferative activity

Ki-67 positivity was observed in the cells of both groups, at the level of the basal and superficial epithelial layers. Only low percentages of Ki-67+ cells were present in control samples (38.31 ± 4.64), while test samples showed high percentages of Ki-67+ cells (55.27 ± 4.58) (Fig.8a-b). This difference was statistically significant (P <0.0001) (Table 1).

Discussion

A successful, accelerated protocol for implant rehabilitation can be affected by several factors: accurate pre-surgical diagnosis and treatment planning, implant macro- and micro-design, and finally adequate fixation and immobility of the implant. The latter have a pivotal role to prevent the risk of micromovements at the implant-bone interface. Rigid splinting seemed to reduce the mechanical stress exerted on implants, and thus influencing the tissue response. Indeed, the loss of implant stability can determine implant failure due to uncontrolled masticatory forces. Consequently, rigid splinting enhances the stability of the prosthetic restoration and the ability to keep the micromovements below the critical threshold.[34]

The endurance of the welding joint is a matter of concern in the development of the intra-oral welding protocol. In a previous study by Degidi et al.[10] the structure of the welding joint was analyzed, and excellent microstructural quality, has been reported. No fracture or radiographically detectable alteration of the welded substructure were found after 12 months of functional loading. The assembling and welding procedure directly in the patients mouth enabled the creation of a precise and passive structure without the need for any correction or further components.[35] Moreover, the intra-oral welding titanium bars described in the study proved to be cost-effective.[35] The reduction of the steps necessary to make a traditional final restoration, also due to the feasibility of this technique with immediate loading, decreased the risks of any eventual damage to the tissues, and thus the risks of recession and bone resorption.[11,36] Therefore, literature has shown

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that immediately loaded implants splinted with titanium bars using intra-oral welding technique can be successful over the long-term.[10, 11, 37,38]

However, the long-term survival of dental implant rehabilitation depends, in part, on the control of bacterial infection in the peri-implant region.[39] Indeed, a positive correlation has been confirmed between oral hygiene and bone resorption at the peri-implant region.[40] Bacterial adhesion to implant or abutment surfaces is a critical issue[41,42] as it can determine the onset of peri-implant mucositis and peri-implantitis. This aspect has not been investigated in implants splinted by titanium bars.

In the present study, the inflammatory infiltrate was mostly present in the soft tissue below titanium bars and its extension was much larger than in controls. The inflammatory infiltrate consisted of T and B lymphocytes, plasma cells, and histiocytes. A modest inflammatory infiltrate is often present in soft tissues below titanium bars and it can also penetrate the submucosa and extend to mucosa. In a study by Degidi et al.[18] investigating inflammation in peri-implant soft tissues around titanium and zirconium oxide healing caps, it has been found that in the titanium specimens a +++ inflammatory infiltrate was present with a larger extension than in zirconium oxide specimens and that these results could be correlated to the higher inflammation processes observed in those tissues. This is in agreement with the results of the present study. However, the present data do not show statistically significant differences in the percentages of T-lymphocytes (CD3+), B-lymphocytes (CD20+) and macrophages (CD68+cells). Statistically significant differences were found in the MVD and in the Ki-67 expression between control and test (P <0.0001). The high intensity of NOS1, NOS3, and VEFG were mainly expressed in test samples, whereas the low intensity of NOS1, NOS3, and VEFG were mostly expressed in controls, with statistically significant differences (P <0.0001).

All these data revealed that the tissues below titanium bars underwent a higher rate of reparative processes, most probably correlated to the higher inflammation processes observed in these tissues. Indeed, inflammation was detected in the areas between dental implants, where the biopsies were taken. On the contrary, these findings do not reflect the condition of the peri-implant soft tissues where the biopsies were not taken and where clinical sign of inflammation were not found. Therefore, inflammation could be due to the adaptation of tissues to the bar supported-prosthesis, especially in the edentulous areas, rather than to plaque accumulation; indeed plaque was not observed at follow-ups.

To the authors best knowledge, this is the first study to investigate the expression of inflammation markers in patients rehabilited by implants splinted with titanium bars. The present histologic and immunohistochemical data demonstrated that the presence of inflammation markers was more prevalent in the soft tissues below bars than in the ones at a distance; however, further long-term studies, where clinical and immunohistochemical data will be collected in parallel, should be conducted for a better understanding of the expression pattern of inflammation markers. Specifically, attention should be focused on the study of the relations between alterations in molecular expression and clinical signs of disease.

Therefore, this study can suggest to submit the patients to a strict protocol of oral hygiene in order to avoid progression of inflammation. Indeed, the use of the titanium bar intra-orally welded to support immediately loaded implants could be a safe technique if supported by a strict clinical protocol of oral hygiene.

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Table 1 Mean values ± standard deviations of immunhistochemical analysis in the control and test groups, the statistical analysis by the means of Wilcoxon mached-pairs signed-ranks test.

	Control (n = 22)		Test (n = 22)		P values
CD 3 (%)	39.09	±8.11	41.36	±8.33	0.4238[ns]
CD 20 (%)	13.40	±3.89	12.95	±3.67	0.7334[ns]
CD 68 (%)	48.40	±9.80	45.68	±9.67	0.3060[ns]
MVD (n/mm^2)	15.22	±4.45	28.18	±3.44	< 0.0001*
VEGF (%)					
High Intensity	37.52	±2.15	55.75	±4.22	< 0.0001*
Low Intensity	62.47	±2.15	44.25	±4.22	< 0.0001*
NOS-1 (%)					
High Intensity	35.59	±7.36	55.09	±4.83	< 0.0001*
Low Intensity	62.63	±2.41	44.90	±4.83	< 0.0001*
NOS-3 (%)					
High Intensity	37.62	±2.82	54.92	±4.56	< 0.0001*
Low Intensity	60.96	±5.37	45.07	±4.56	< 0.0001*
Ki-67 (%)	38.31	±4.64	55.27	±4.58	< 0.0001*

Percentage (%) of CD 3 (T cells), CD 20 (B cells), CD 68 (macrophages/histiocytes), Ki-67+ cells.

Percentages (%) of areas occupied by high and low intensities of expression of VEGF, NOS1 and NOS3.

MVD values are expressed as number of microvessels per square millimeter (n/mm^2).

Significance of difference between groups was measured using Wilcoxon matched-pairs signed-ranks test ($P < 0.05$). The percentages are expressed mean ± standard deviations.

* significance

[ns] not significant

Fig. 1 Clinical images showing a case of intra-orally welded implants **(a)** before surgery; **(b)** after implant placement and bar welding; **(c)** delivery of a provisional functional prosthesis; **(d)** delivery of a metallo-ceramic prosthesis.

Fig. 2 Image showing how the extension of the inflammatory infiltrate was evaluated: each specimen was divided into 3 areas: the 1/3, 2/3, 3/3.

Fig. 3 In the control group **(a)** the inflammatory infiltrate was much lower and less extended than in test group **(b)**. H & E 10×

Fig. 4 (a) In the test group several small and large microvessels were present in the submucosa. **(b)** same field showing how the MVD count was undertaken.

Fig. 5 The intensity of VEGF expression was different between control **(a)** and test **(b)** groups. The control group cells mainly showed low intensity (green) of VEGF expression **(c)**, while in the test group high intensity (red) of VEGF expression were prevalent **(d)**. Strep ABC VEGF 20×

Fig. 6 In both control **(a)** and test **(b)** groups NOS1 was expressed in all the examined tissues, even though the intensity of expression was different. The control group **(c)** presented lower values of high intensity (red) of expression than test group **(d)**. Strep ABC NOS1 20×

Fig. 7 NOS3 was expressed in all the examined tissues of control **(a)** and test **(b)** groups. The intensity of the expression, however, was different between the two groups. It was observed that the cells of control group **(c)** showed higher values low intensity of expression (green) than test group cells **(d)** which presented higher values of high intensity (red) of expression than the cells. Strep ABC NOS3 20×

Fig. 8 Ki-67 positivity was detected in the basal cells of epithelium in control samples **(a)**, while test samples showed several Ki-67+ cells located in the basal and more superficial layers of epithelium. Strep ABC Ki-67 20×

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Fig. 1

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Fig. 2

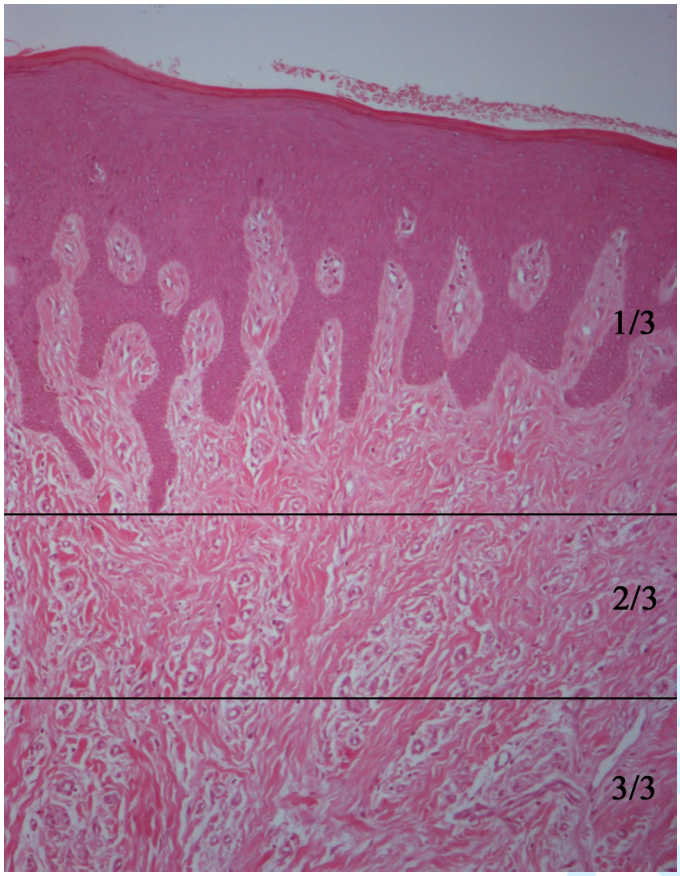

Fig. 3

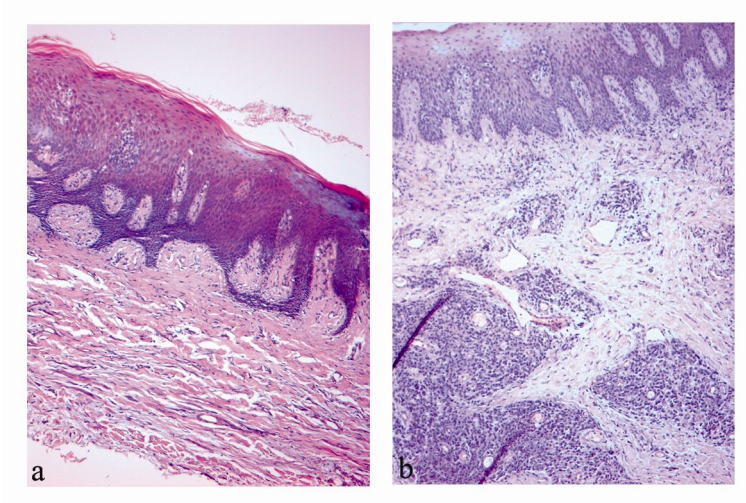

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Fig. 4

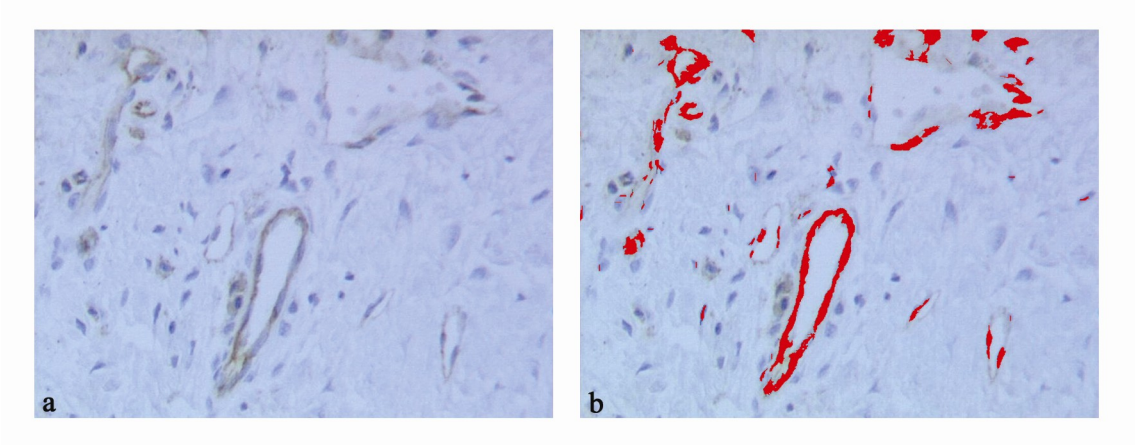

Fig. 5

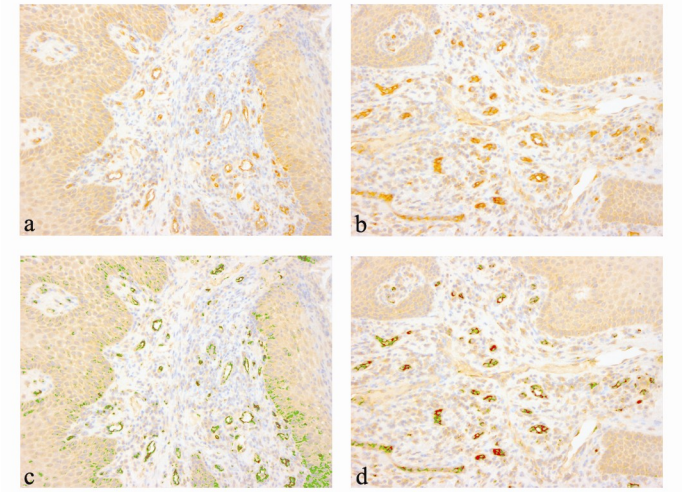

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Fig.6

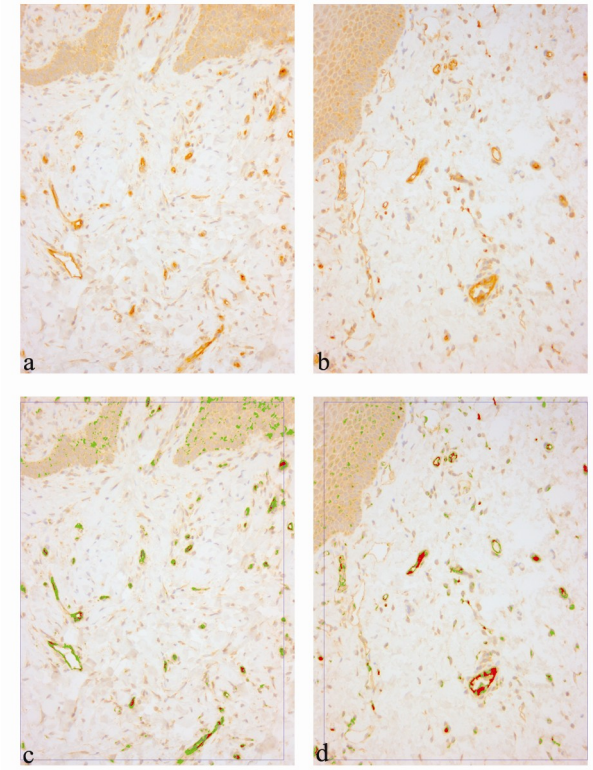

Fig. 7

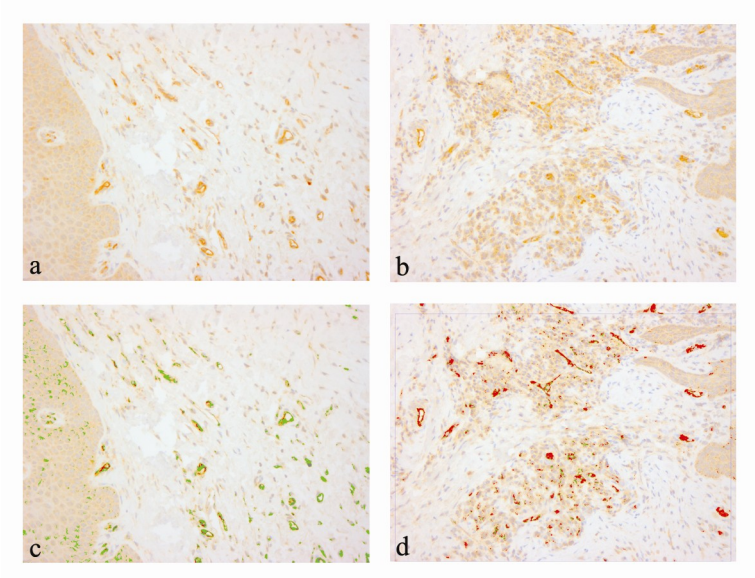

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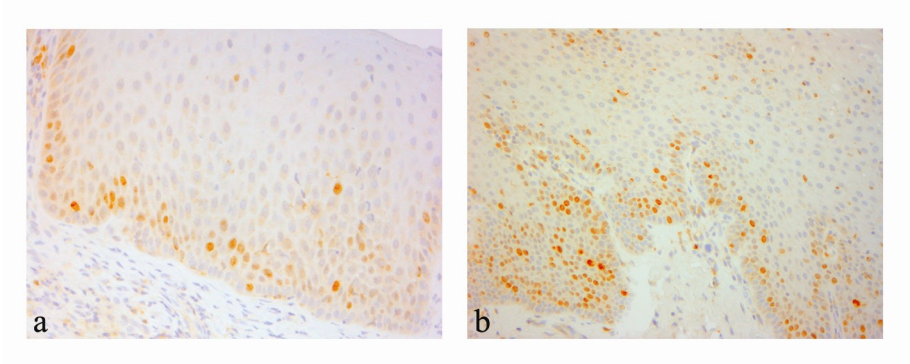
Fig. 8



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