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Expression of bone-regulatory proteins in human valve allografts

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Running Title: Bone-regulatory proteins and valve allografts

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Abstract:
Introduction: Calcific degeneration of valve allografts (VA) reduces their longevity. We hypothesized that VA calcification was related to an ossification process which involved the expression of bone-regulatory proteins. **Methods**: We studied 15 VA that were explanted at the time of surgery for dysfunction. VA were analyzed and compared to normal aortic valves (n=20). **Results**: All the VA (5 aortic, 10 pulmonary) exhibited heavy calcification and important fibrosis. Immunohistochemistry studies demonstrated that the bone specific transcription factor, Cbfa-1, was expressed by stromal cells. Bone alkaline phosphatase (ALP) was expressed in calcified regions. Immunostaining for alpha-smooth muscle actin (a-SM actin) was increased in VA compared to normal valves and formed in 6 of the 15 valves cellular clusters at the proximity of calcified nodules. In VA osteopontin and osteonectin were expressed by stromal cells, whereas osteocalcin was closely associated with the calcified regions. Furthermore, analysis of the bone-regulatory proteins that control bone resorption revealed that RANKL/RANK/OPG were differentially expressed in calcified VA and normal valves. Normal valve leaflets expressed osteoprotegerin (OPG), whereas no or faint expression of OPG was documented in calcified VA. RANKL and RANK were not detected in normal valves, whereas calcified VA expressed RANKL and RANK. **Conclusion**: These data suggest that calcification of VA involves an ossification process, which relies on a tight control of bone-regulatory proteins expression. The expression pattern of the RANKL/RANK/OPG system suggests that it might have a regulatory role not only in osteoclastogenesis but also in the calcification of human VA.

Keywords: Valve allograft, bone-regulatory protein, ossification, osteoprotegerin.

Introduction

Clinical performances of valve allografts (VA) are good, however the longevity of these valve substitutes is impeded by the calcification process, especially in young patients.[1] Calcification of VA is associated with structural valve failure that eventually leads to reoperation.[2] In the past heterotopic calcification was considered a passive mechanism involving the deposition of calcium in degenerative tissues. More than a decade ago the works of Demer's and Giachelli's groups have established that calcification of vascular structures was not the result of a mere passive deposition of calcium but was rather related to an active cellular process.[3 4] Recently, calcification of valve tissue was also linked to an active transformation of resident interstitial cells toward bone forming cells.[5] Calcification of VA has been related to immune mechanisms and inflammation within the grafts.[6] However, regulation of the ossification process has not been thoroughly described in VA.

In bone, ossification is an exquisitely regulated process that is under the control of numerous molecules including, phosphatase, growth factors, extra-cellular matrix (ECM) proteins, and matricellular proteins. Osteoblast differentiation is regulated through the expression of the bone specific transcription factor, Cbfa-1, which also regulates the rate of bone matrix deposition by differentiated osteoblasts.[7] Proper calcification of bone tissue is dependent on the expression and activity of alkaline phosphatase (ALP).[8] Furthermore, the biomineralization process is regulated through the production of non-collagenous bone proteins including the matricellular proteins, which are secreted biomolecules that interact with cell-surface receptors, ECM and growth factors but do not in themselves play a direct structural role. Osteonectin and osteopontin are matricelullar proteins that regulate the communication between cells and the ECM and are involved in the control of the ossification process.[9 10] Osteocalcin is the most abundant non-collagenous bone protein but its role in the regulation of the biomineralization process is not completely elucidated.[11] Recently, a group of molecules from the tumor necrosis factor (TNF)-related family has been shown to play a key role in skeletal and vascular mineralization. RANKL (receptor activator of nuclear factor κB ligand) is expressed by T cells, and osteoblasts especially in regions of active bone remodelling. RANKL binds to RANK (receptor activator of nuclear factor κB) on the surface of monocyte/macrophages lineage cells, including osteoclasts and promotes cell survival and activation.[12] Osteoprotegerin (OPG) is a secreted protein, which regulates bone mass and osteoclast activation by binding to RANKL. Mice deficient for OPG develop osteoporosis and severe vascular calcification.[13] OPG is secreted by the vascular wall and might participate to the protection of blood vessels against heterotopic calcification. In this study we hypothesized that calcification of human VA leaflets involved the production of bone regulatory proteins including the RANKL/RANK/OPG system.

Materials and Methods

Patients

We examined 15 cryopreserved VAs that had been implanted for periods ranging from 2 to 16 years (median 8.7 years). There were 5 aortic and 10 pulmonary VAs implanted in the pulmonary position for reconstruction of the right ventricular outflow tract (RVOT). The mean age of patients was 17±6 (range 2-44) years and proportion of male to female was 9/6. The mean age of VA donors was 21.4 years (range of 12 to 33). Reasons for explantation were stenosis in 9 patients and a predominant regurgitation in 6 patients. For controls, we have selected 20 heart transplant recipients who had no valve disease, from whom the aortic valve was obtained at the time of transplantation. Samples were taken at the time of surgery. Cusps including the conduit were immediately transported to the laboratory in cold HEPES solution. Tissues were decalcified in Cal-Ex (Fisher, Nepean, Ont, Can) for 24 hours and then one cusp was fixed in formaldehyde 10% for histological processing and one cusp was embedded in optimum cutting temperature (OCT) compound (TissueTek, Miles Laboratories, Elkhart, Ind, USA) and frozen in liquid nitrogen for immunohistological analysis.

Histological analysis

Decalcified and fixed tissues were processed for routine paraffin embedding. Valve samples were excised vertically to the base at the midpoint. Sections of 5 μm were obtained and stained with hematoxylin-eosin (H&E) and trichrome Masson. Calcification of VA was determined using the alizarin red coloration (Sigma, Oakville, Ont, Can).

Immunohistological analysis

Immunohistology was performed in cryostat sections. Immunohistological analysis was performed using the following mouse Mab: anti-collagen type I (Sigma, St-Louis, MI, USA), anti-bone alkaline phosphatase (ALP) (R&D systems, Mineapolis, MN, USA), anti-alpha smooth muscle actin (a-SM actin) (Sigma, Oakville, Ont, Can), anti-osteonectin (US biological, Swampscott, Mass, USA), anti-osteocalcin (US Biological, Swampscott, Mass, USA), anti-osteoprotegerin (OPG) (Chemicon Int, Temcula, Ca, USA), anti-RANKL (Imgenex, San Diego, Ca, USA), anti-RANK (R&D Systems, MN, USA). The following polyclonal antibodies were used: rabbit anti-Cbfa-1 (US Biological, Swampscott, Mass, USA) and goat anti-osteopontin (R&D Systems Inc, MN, USA). Slides were then incubated with a biotin-conjugated anti-mouse immunoglobulin antibody (Jackson Immuno Research, Westgrove, PA, USA), or an anti-goat immunoglobulin antibody (Biocan Scientific, Mississauga, Ont, Can) or an anti-rabbit immunoglobulin antibody (Biocan Scientific, Mississauga, Ont, Can), followed by HRP-conjugated streptavidin and ABC substrate (Dako, Mississauga, Ont, Can). Tissue sections were counterstained with hematoxylin. Secondary antibodies without the primary antibody were used as controls in all the experiments and in this study isotype-matched irrelevant antibody were not used.

Results

Macroscopic findings in VA valves reveal that all the conduits were heavily calcified, whereas valve cusps presented two different patterns of calcification. The VA cusps from patients with VA stenosis showed severe calcification, whereas the VA cusps from patients with a regurgitant VA showed less calcification but presented leaflets retraction (figure 1 a,b). Histological analysis showed that all the VA cusps were calcified at the cusps insertion (1c). In eleven patients calcified nodules were present not only at the cusp insertion but extended through the leaflet matrix (figure 1 d). Trichrome Masson staining and immunostaining for collagen type I showed that VA were densely infiltrated with collagen compared to normal valves (figure 1e, f, g, h).

In VA we found that a-SM actin immunostaining was increased when compared to normal valves. Moreover in 6 VAs, a-SM actin positive cells formed clusters at the proximity of calcified nodules (figure 2 a,b). The bone transcription factor, Cbfa-1, was not found in normal valves, whereas it was expressed by stromal cells within VAs (figure 2 c). In normal valves the ossification marker, ALP, was immunodetected in the fibrosal layer while it was increased and located to calcified areas in VAs (figure 2 d, e). We have found that osteocalcin, osteopontin and osteonectin were not present in normal valves whereas they were detected in VAs. Immunohistochemical studies demonstrated that osteocalcin was closely associated with calcified areas, whereas osteopontin and osteonectin were immunodetected in stromal cells within leaflet matrix (figure 2 f, g, h).

Analysis of normal valves revealed that their cusps had a strong immunoreactivity for OPG (figure 3 a) whereas RANKL and RANK were not detected. As opposed to normal valves, VAs had no or faint expression of OPG (figure 3b) whereas RANKL and RANK were immunodetected (3 d, f). RANKL was detected as an extra-cellular protein and it was not detected in stromal cells (3 e). RANK was detected in VAs and immunolocalized to stromal cells that on occasion formed cellular aggregates (figure 3 f).

Discussion

VA structural failure is closely associated with graft calcification. Calcification of extra-osseous tissue once considered a passive process is actually viewed as an active cellular mechanism involving the production of bone specific proteins. Leaflets are composed of valve interstitial cells represented by fibroblasts and myofibroblasts. Myofibroblasts expressing a-SM actin are active synthetic cells that are involved during active tissue remodelling.[14] We have observed that normal valves were composed of mainly fibroblasts with the presence of occasional myofibroblasts expressing a-SM actin, which were dispersed within the leaflet matrix. On the other hand we have found that VA valves were composed of myofibroblasts which formed in some grafts cellular clusters at the proximity of calcified regions. In VAs, the presence of a-SM actin positive cells at the proximity of calcified regions might reflects the active synthesis of ECM in areas where calcified matrix is deposited. In vitro isolated interstitial cells calcify when cultured in appropriate conditions, indicating the plasticity of this cellular population and their potential implication in the extra-osseous ossification process. Isolated valve interstitial cells loose the a-SM actin marker and acquire a bone phenotype during mineralization.[5] It has been demonstrated in an experimental model that calcification of aortic allografts is associated with the transformation of a-SM actin positive cells into chondrocytes followed by an endochondral ossification, indicating that a-SM actin positive cells are precursor of bone forming cells within allografts.[15]

VA leaflets were associated with dense deposition of collagen type I along with calcification that started at cusp insertion with extension within the leaflet matrix. Ossification is closely associated with the synthesis of collagen type I, it is the most abundant protein in osseous tissue, and it is necessary for proper mineralization of bone.[16]

Fibrosis and retraction of leaflets were a predominant feature of regurgitant VAs, whereas stenotic VAs were characterized by extensive leaflet calcification. Factors that drive a pro-fibrotic and a retraction process of VA leaflets compared to a predominant calcifying response are unknown but potential mechanisms related to a differential production of growth factors might be involved.

Maturation of osteoblasts involves the coordinate expression many genes and transcription factors. Differentiation of precursor cells toward osteoblasts is marked by the expression of bone specific proteins and transcription factors such as Cbfa-1.[17] We have observed in VAs a population of cells expressing the bone transcription factor Cbfa-1, indicating the presence of cells with a bone-like phenotype within VAs. In extra-osseous mineralized tissues such as calcified atherosclerotic aorta, bone matrix proteins along with their specific bone transcription factors such as Cbfa-1 have been detected.[18] The transcription targets of Cbfa-1 include bone sialoprotein, collagen type I and osteopontin.[19]

Alkaline phosphatase (ALP) long recognized as an osteoblastic marker, is a membrane associated enzyme that converts organic phosphate into orthophosphate in order to form hydroxyapatite crystals.[20] A high level of bone ALP activity is necessary to ensure an adequate mineralization of bone since a genetic deficiency in the activity of this enzyme is associated with impaired skeletal development. In vitro, blockage of ALP inhibits the formation of calcified nodules in isolated leaflet interstitial cells.[5] In the present study, normal valves expressed ALP within the fibrosal layer, whereas VAs had an increased expression that was associated with calcified regions. Although the role of ALP in normal valves is still unknown, it is believed that increased enzymatic activity in calcified tissue is necessary in order to achieve mineralization.[21]

Ossification and control of the mineralization process is complex and partially understood. A large number of reports have documented the presence of three non-collagenous matrix proteins, osteonectin, osteopontin, and osteocalcin in bone and in mineralized tissues outside the skeleton.[22] Osteonectin is a calcium binding protein that has an affinity for collagen, suggesting that it might support the formation of hydroxyapatite. In bone osteonectin promotes osteoblast formation and survival supporting the concept that it is a positive regulator of bone formation.[23] Osteopontin is an acidic phosphoprotein with a hydroxyapatite binding site suggesting that it regulates the calcification process. It accumulates in injured blood vessels and in calcified areas. It has been demonstrated that osteopontin has a potent inhibitory action on the calcification process.[24] Osteocalcin is a vitamine K-dependent protein that strongly inhibits hydroxyapatite nucleation. Osteocalcin expression has been documented in mineralized areas of atherosclerotic plaques and aortic stenosis.[25] We have found in VAs that osteonectin and osteopontin were immunodetected in stromal cells while osteocalcin was detected in calcified areas. The presence of non-collagenous bone proteins within VAs suggests that the mineralization process of allografts is similar to bone formation.

Recently discovered the RANKL/RANK/OPG system appears to mediate important interactions between the immune system, bone tissue and blood vessels. Animals deficient for OPG develop osteoporosis and severe vascular calcification supporting the hypothesis that OPG play a role –although undefined at the moment- in the protection against vascular calcification.[26] Furthermore, systemic administration of OPG in rodents prevented warfarin and vitamin D-induced extra-osseous calcification.[27] Tissue expression of OPG is greatly reduced in calcific aortic stenosis, reinforcing the hypothesis that modulation of OPG in peripheral tissue might be involved in the regulation and the control of extra-osseous calcification.[28] We have immunodetected OPG in normal aortic leaflets where it was abundant, whereas in VAs, expression was faint or absent, suggesting that OPG might have a protecting role against calcification in allografts. RANKL and RANK were immunodetected only in VAs, as a diffuse extra-cellular immunomarking for the former while in the latter, expression was cellular. Kaden et al. have demonstrated in isolated valve interstitial cells that RANKL induced matrix calcification and increased expression of Cbfa-1, osteocalcin and ALP.[28] RANKL is initially synthesized as a membrane anchored molecule which can be cleaved as a soluble molecule by the metalloprotease-disintegrin TNF-α convertase (TACE).[29] In VAs the immunomarking for RANKL suggested that it was secreted, but its cellular origin and the mechanisms for its production remain to be answered. The membrane bound protein, RANK, is expressed by osteoclasts, chondrocytes and dendritic cells. Osteoclast-like cells expressing RANK have been described in calcified atherosclerotic plaque, suggesting that mineral resorption might be a defensive mechanism of the arterial wall.[30] Therefore, differential expression of the RANKL/RANK/OPG system in VAs suggests that along with its implication in the control of bone resorption, this pathway might be involved in the calcification of allografts.

In this study, expression of bone proteins has been documented using immunohistochemistry studies as the sole technique, which might introduce some limitations to the interpretation of the results. However, the results obtained from other studies using either immunohistochemistry studies or other techniques such as mRNA amplification in calcified vascular tissue or aortic stenosis have yielded comparable results.[22 25] Calcification of VAs long recognized as an important mechanism for structural failure, is actually perceived as an active cellular process involving numerous and complex interactions between growth factors, bone-regulatory proteins and the ECM. To our knowledge this is the first study to document the differential expression of the RANKL/RANK/OPG system in the calcification process of human VA. Although not fully understood, the expression pattern of bone-regulatory proteins including the RANKL/RANK/OPG system in VAs suggests that an active cellular mechanism related to bone ossification is involved in the mineralization process of allografts. Understanding the mechanisms involved in the mineralization process of VAs might help to design new therapeutics aim to prevent VA structural failure.

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Figure legends

Figure1 : Macroscopic inspection of explanted VA reveals that all the conduits were mineralized, a) leaflets were heavily calcified in patients with a predominant stenosis leaving only a small aperture for blood passage (arrow), b) whereas in patients with a regurgitant allograft leaflets appear retracted; c) small nodules of calcification were localized at cusp insertion as seen on H&E examination (400x); d) alizarin red coloration showed extension of calcified foci into the leaflet matrix (400x); e) trichrome Masson staining in normal valves showed deposition of collagen (green) in the fibrosal layer (100x), f) whereas we found in valve allografts dense deposition of collagen with thickening of leaflets (100x); g) immunostaining for collagen type I demonstrated the presence of collagen in normal valves (100x), h) that was increased in leaflets of valve allografts (100x).

Figure 2 : a) Immunostaining for a-SM actin in normal valves showed dispersed positive cells within the matrix, b) whereas in valve allografts we found numerous a-SM actin positive cells forming cellular clusters (200x); c) in valve allografts stromal cells were positive for Cbfa-1 (400x); d) normal valves expressed bone alkaline phosphatase (ALP) localized to the fibrosal layer, while e) in valve allografts ALP immunostaining was increased and localized with calcified areas (200x); f)osteocalcin was only immunodetected in allografts and localized to calcified areas, whereas, g) osteopontin and h) osteonectin localized to the stromal cells (200x).

Figure 3: a) OPG was immunodetected in normal leaflets b) whereas it was not detected or appears as a weak expression in valve allografts (200x); C) RANKL was absent in normal leaflets d) but it was immunodetected in valve allografts (100x) e) as a soluble protein (400x); f) RANK was not detected in normal valves (not shown) but it was immunodetected in stromal cells of valve allografts (400x).

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Figure 1

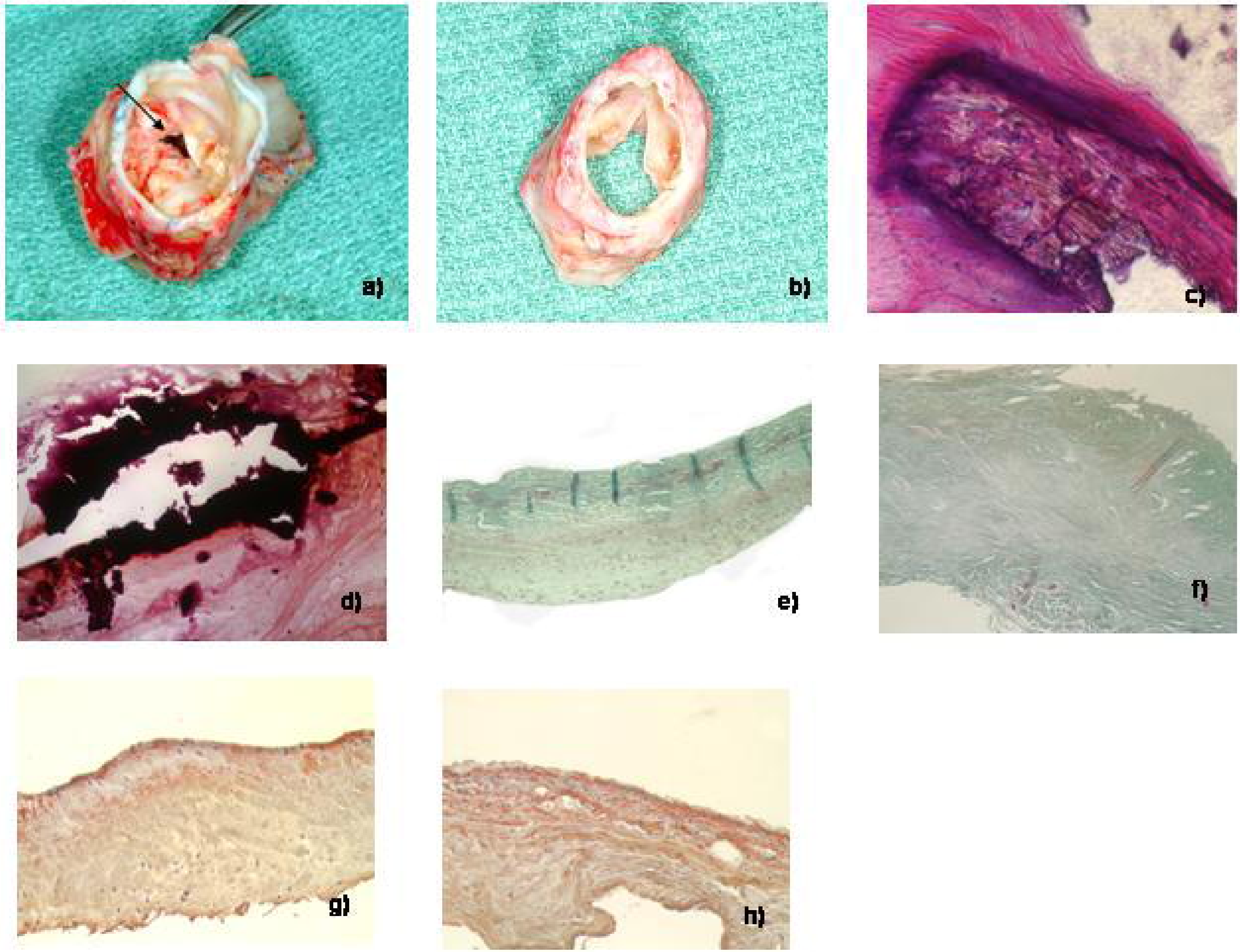


Figure 2

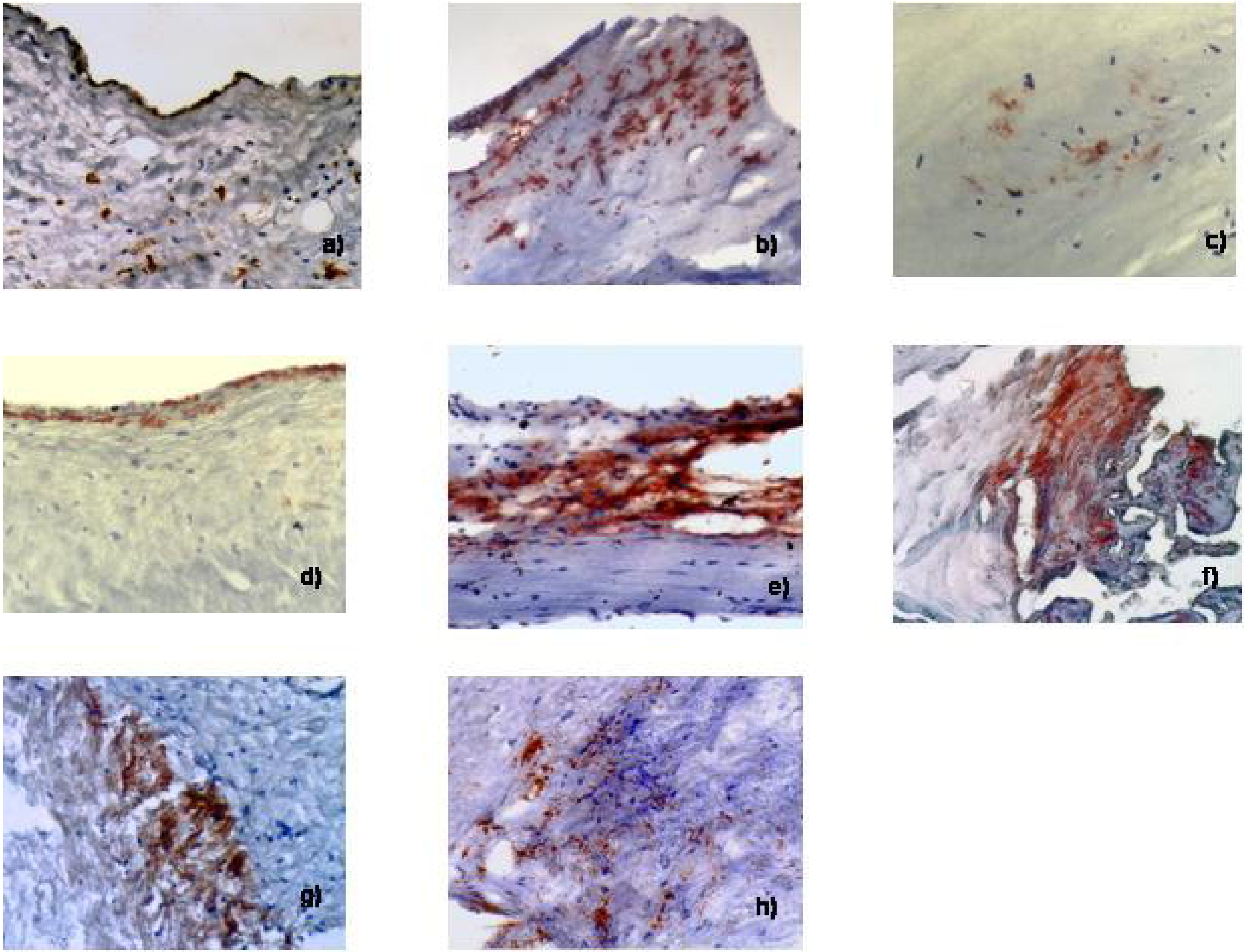
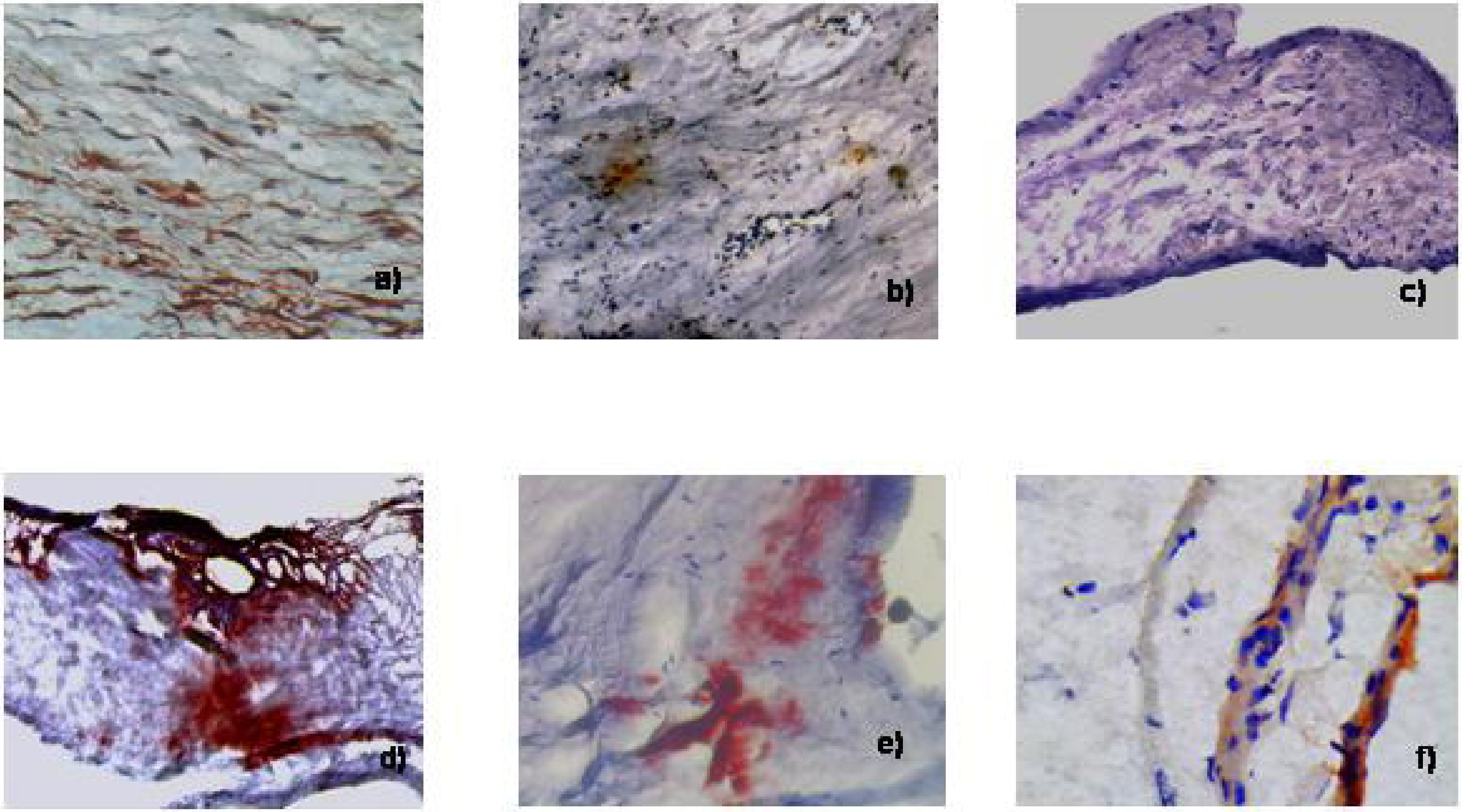


Figure 3



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