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Porcine coronary arteries: immunohistochemical profile of TNF-α, IL-1β, TGF-β1 and ICAM-1

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ABSTRACT

In our study we used immunohistochemical technique to demonstrate the presence of the cytokines TNF-α, IL-1β, TGF-β1 and ICAM-1 in porcine coronaries even in physiological conditions. Inflammatory cytokines are polypeptide mediators which act as a communication signal between immune system cells and other types of cellsin different organs and tissues, both in human and pig

coronary circulation. Our results show that pro-inflammatory cytokines TNF-α, IL-1β, TGF-β1 and ICAM-1 are also present in the medium tunica of the coronary arteries under physiological conditions. These results may be compared with those found in coronary atherosclerosis, where the increase in TNF-α has a dramatic effect on the function of the left ventricle, and the high value of IL-1 correlates directly with the extent of myocardial necrosis. In our study we observe the damage and activation of endothelial cells, this induces endothelial dysfunction by accumulation and oxidation of LDL (Low Density Lipoproteins). The formation of oxidized LDL (ox LDL) could play a central role in the amplification of the inflammatory response causing an increased expression of pro-inflammatory cytokines which promotes leukocyte recruitment in the intimal layer. These leukocytes, after the adhesion to the endothelium, penetrate the intimate tunic. Therefore inflammatory processes promote the onset and evolution of atheroma and the development of thrombotic complications.

Key words: porcine coronary arteries, cytokines, IL-1beta, TNF-alfa, TGF-beta1, ICAM-1

INTRODUCTION

Cytokines are proteins produced by various types of cells in response to a stimulus, they have autocrine or paracrine effects and led the cells of the immune system to communicate with each other. Many cytokines are pleiotropic, because they act on a large variety of cells and tissues [1, 2]. Cytokines play an important role in cardiovascular disease by inducing inflammation, cell proliferation and apoptosis. Activated macrophages are known to be a major source of inflammatory cytokines. Previous studies have shown that cells present in the vascular wall, such as vascular smooth muscle and endothelial cells, produce different types of cytokines that are involved in cardiovascular disease [3-5]. Coronary atherosclerosis represents the main cardiac manifestation of a systemic pathological process initiated and fueled by cellular and humoral mechanisms of an inflammatory nature [6]. Most cardiac pathologies such as acute myocardial infarction and its complications are the result of the action of a complex inflammatory network that culminates and is concentrated in the event acute but involves the entire body and in particular the cardio-vascular system [7]. The atherosclerotic process begins with the accumulation of LDL-cholesterol in the intestine with consequent endothelial damage, which favors inflammation, which is responsible for the expression of adhesion molecules on the cell membrane and the secretion of biologically active and chemotactic substances such as cytokines, growth factors and free radicals; these substances

favor the recall and subsequent infiltration of leukocytes, with transformation of monocytes into macrophages. The secretion of inflammatory cytokines and some growth factors favor the proliferation and migration towards the intima of the smooth muscle cells of the middle layer, promoting the formation of the fibrolipidic plaque [8-10]. The formation of atherosclerotic plaques determines the onset of coronary heart disease. They form on the inner walls of the cardiac arteries following the migration of SMC and the production of components of the extracellular matrix. These pro-inflammatory events recruit immune cells into the plaques causing severe inflammation and thrombosis [11]. The precise molecular mechanisms responsible for the transformation of an atheroma into an atherosclerotic plaque remain unknown, based on the theory that inflammation is the main trigger we decided to carry out a preliminary study on porcine coronary arteries.. The porcine heart offers really a good resemblance to the human heart, especially about coronary arteries and hemodynamics. Moreover, the wide social acceptance of their use, the similarity of their coronary circulation to that of humans, the approximation of their hemodynamic values and the size of the heart, make this animal model ideal for experimental studies [12,13]. In the present study we studied the expression of some cytokines that are supposed to be involved in the cascade mechanisms after vascular damage.IL-1 and TNF-α practically act on all cells and tissues and are defined as “primary inflammatory cytokines”, because they are capable of setting in motion the entire cascade of mediators of the inflammatory response [14,15]. TGF-β1 is the most widespread cytokine present in endothelial cells, connective tissues and blood. ICAM-1 (Intercellular Adhesion Molecule-1) is a member of the immunoglobulin super family, including antibodies and T-cell receptors, it is present in low concentrations in the membranes of leukocytes and endothelial cells. ICAM-1 release may be induced by Interleukin-1 and TNF-α and it is expressed by vascular endothelium, macrophages and lymphocytes. Investigation of the mechanisms of atherosclerosis determined that inflammation plays a central role in the development, progression and outcome of acute coronary syndrome. Many scientific works demonstrate the role of some cytokines in acute coronary syndrome: IL-8, IL-10, IL-18, IL-2, TNF-α, interferon γ are involved in this clinical condition [14-20]. The inflammatory theory of Russell Ross [21] concerns the mechanism by which cardiovascular risk factors evoke a restorative response from the vascular wall. This mechanism consists of a response that begins by endothelial activation, a purely functional alteration, and then it progresses and stabilizes by the formation of an atherosclerotic plaque, a real morphological alteration. This key concept focuses scientific interest in the actors of the inflammatory response, in particular the leukocytes and the mediators that modulate and support this process.This theory is supported by experimental data and simultaneous elevation, in case of coronary atherosclerosis, of the markers of inflammation, including also some inflammatory cytokines with proven implications on prognosis. In various animal models of atherosclerosis, signs of inflammation have been found

together with lipid accumulation in the wall of the arteries. In an experimental diabetic model obtained from pigs with streptozotocin-induced diabetes, an increased production of superoxide in coronary media and adventitia, due to increased NADPH oxidase activity, has been described [22]. Diabetes-induced oxidative stress resulted in an inflammatory response in the adventitia (increased expression of interleukin-6, TNF-α, monocyte chemotactic protein-1, vascular cell adhesion molecule-1 -VCAM-1-) and in the media tunic (VCAM-1 only). An enhanced redox state is accompanied by the upregulation of inflammatory cytokines (IL-6 and TNF-alpha), chemokines (MCP-1), and adhesive molecules (VCAM-1).VCAM-1 and inter-cellular adhesion molecule (ICAM) are highly expressed around adventitial vasa vasorum. Expression of proinflammatory cytokines such as interleukin 6 (IL-6) and IL-8 has been reported to be increased in acute coronary syndromes[23,24]. This observation suggests additional vascular portals for inflammatory cells. In fact, several inflammatory cells (lymphocytes and mast cells for instance) have been noted in the adventitia after fatal acute coronary syndromes or intractable vasospasm [22,25]. In our study we demonstrate the presence of cytokines TNF-α, IL-1β, TGF-β1and ICAM-1 in the porcine coronary arteries even in physiological conditions.

MATERIALS AND METHODS

Sampling

A total of 22 swine hearts (Sus scrofa domestica, Great white Polish pigs, all males, 18–30 weeks old, 80–140 kg of weight) were dissected up to one hour after commercial animal slaughter. No animals were sacrificed deliberately for this studyand all samples were originally intended as obtained for use in the food industry. All hearts were normal with no congenital heart defects and were obtained from healthy animals that had no recognizable diseases. The hearts were obtained intact, washed until free of blood and blood clots using a saline solution. Specimens of coronary arteries (left carotid artery, left carotid right and circumflex coronary arteries) have been harvested, fixed in formalin, embedded in paraffin and then cut in serial sections (7 um of thickness).

Immunohistochemistry (IHC)

The immunohistochemical analysis was performed using the ABC/HRP technique (avidincomplexed with biotinylated peroxidase) on 7 μm thick paraffin sections that were cut using a rotative microtome. These sections were deparaffinized and hydrated through decreasing ethanol series to distilled water, then subjected to microwave irradiation and immersed in citrate buffer (pH= 6) twice for 5 minutes each time. Subsequently, endogenous peroxidase activity was

quenched using 0.3% hydrogenous peroxide in methanol for 30 minutes. To evaluate the immunolocalization of TGF-β_1, IL-1β, TNF-α and ICAM-1 the following antibodies were employed: i) rabbit anti-TGF-β_1 polyclonal antibody (dilution 1:50; cat no. sc-130348;Santa Cruz, CA, USA); ii) rabbit anti-IL-1β polyclonal antibody (dilution 1:50; cat no. sc-12742;Santa Cruz Biotechnology, Santa Cruz, CA, USA); iii) mouse anti-TNF-α monoclonal antibody (dilution 1:100; cat no. sc-133192;Santa Cruz Biotechnology); iiii) Mouse anti-ICAM-1 monoclonal antibody (dilution 1:25; cat no. sc-107; Santa Cruz, CA, USA). Incubation with the primary antibodies was performed overnight at 4º C. Optimal antibody dilution and incubation times were assessed in preliminary experiments. As negative control, the primary antibodies were omitted. After exposure to the primary antibodies all slides were rinsed twice in phosphate buffer (pH=7.4) and incubated for 1 hour with the appropriate secondary biotinylated antibody at the final dilution of 1:200. The secondary biotinylated antibodies against rabbit and mouse immunoglobulins were purchased from Abcam (biotinylated goat anti-mouse antibody and biotinylated goat anti-rabbit antibody). The slides were then incubated with peroxidase-conjugated avidin (Vector laboratories, Burlingame, CA, USA, Vectastain Elite ABC kit Standard*PK 6-100) for 30 min. Slides were washed in phosphate buffer (pH=7.4) and treated with 0.05% 3,3-diaminobenzidine (DAB) and 0.1% H_2O_2. Finally, sections were counterstained with Mayer's hematoxylin and dehydrated rapidly. The staining assessment was made by three experts. Negative control experiments were carried out: i) by omitting the primary antibody; ii) by substituting the primary antibody with an equivalent amount of non-specific immunoglobulins; iii) by pre-incubating the primary antibody with the specific blocking peptide (antigen/antibody = 5 according to supplier's instructions). The staining assessment was made by two experienced observers in light microscopy. Immunoreactivity of TGF-β_1, IL-1β, TNF-α and ICAM-1 was assessed in all samples.

The intensity of the immune reaction was assessed microdensitometrically using an IAS 2000 image analyzer (Delta Sistemi, Rome, Italy) connected via a TV camera to the microscope. Twelve 100μm^2 areas were delineated in each section by measuring the diaphragm. The system was calibrated taking the background obtained in sections exposed to non-immune serum as zero.

Statistical analysis

All experiments were performed on at least three occasions in duplicate.
We divided the pigs into two groups:
-group1 pigs 18–24 weeks old;
-group 2 pigs 24–30 weeks old.

To perform the statistical analysis we compared the percentage of expression of each cytokine analyzed in the two groups. Comparisons between the two groups were analyzed using the

unpaired Student's t test. Quantitative data of the intensity of immune staining were analyzed statistically by analysis of variance (ANOVA). Data are expressed as mean + SEM.A value of *P-value<0.05* was considered significant.

RESULTS

On macroscopic examination, no obvious coronary lesions were found in the analyzed segments. Positive areas for each immunohistochemical stain were measured. IL-1β protein was detected in the adventitial vessel endothelium in 12 of 12 specimens, IL-1β was present in macrophages (mainly foam cells) of 10 of 11 specimens of coronary arteries. Immunostaining for IL-1β showed a strong positivity in endothelial cells and in nuclei of the middle tunic smooth muscle cells (Figure 1A-1B). The presence of IL-lβ in coronary arterial wall cells was studied by histochemistry to determine the reproducibility of the histological grading, eight blocks had multiple sections cut. The other cytokines analyzed, such as TGF-β1, ICAM-1 and TNF-α, also showed positive reaction mainly in the nuclei of the smooth muscle cells of the media and in the endothelial cells (Figures 2A-2B, 3A-3B and Figures 4A-4B). Our results show also that, under physiological conditions, the pro-inflammatory cytokines IL-1beta, TGF-β1, ICAM-1 and TNF-α are already present in the medium tunic of the coronary arteries. In our study high constitutive expression of TGF-β is noted in coronary arteries, however, the in vivo relevance of this observation is unclear. Semi-quantitative analysis of inflammation was done by scoring the number of infiltrated inflammatory cells in sections of the vessel wall. In our study we observed that the tipic morphology of the coronary arteries was preserved in the entire vessels analized, in presence of inflammatory cytokines was observed a significant increase in neointimal thickness, neointimal area, the neointima was composed almost entirely of cells with little intercellular matrix.

Quantitative data of the intensity of immune staining, were analyzed statistically. We have divided the pigs into two groups, In our analysis, there is no significant difference between the two groups. As shown in Table I, the immunohistochemical analysis did not reveal any significant differences in the expression of the cytokines analyzed between the two groups.

DISCUSSION

Cytokines play an important role in diseases of the cardiovascular system by inducing inflammation, cell proliferation and apoptosis. It is well known that activated macrophages are a major source of inflammatory cytokines [25]. The intimal layer is the site of the earliest vascular atheromatous alterations. The pathogenetic development of these lesions is still not entirely clear today. It is believed that the endothelium plays a fundamental role in the early development of plaque. The most accredited hypothesis places a reaction to intimal layer damage at the basis of this

process. In this case, atherosclerosis would represent an inflammatory response of the vascular wall. The location in the intimal layer of low density lipoproteins which, adhering to the proteoglycan layer [26] of this tunica, tend to form aggregates that are more difficult to remove from this site [27], represents a crucial phase of the inflammatory response. Their prolonged accumulation in the tunica intima exposes lipoproteins to the oxidative stress whichlater induces chemical modifications that are considered crucial for the progression of the atherosclerotic process [28]. Oxidized liporoteins are able to activate the local production of cytokines that stimulate the expression of adhesion molecules and growth factors from some of the endothelial cells. A functioning endothelium may resist to the adhesion and migration of circulating inflammatory cells. In atherosclerotic endothelial dysfunction, on the other hand, there is an increased expression of molecules such as VCAM-1 (Vascular Cells Adhesion Molecule-1), ICAM-1 (Intercellular Adhesion Molecule-1) and selectins (E-selectin, P-selectin). It is noteworthy that VCAM-1 is a receptor for an integrin (VLA-4) which is selectively expressed on cells that are early found in the native atheroma as monocytes and T lymphocytes. VCAM-1 and ICAM-1 promote the adhesion and immobilization of circulating cells [29], while the role of selectins (particularly P-selectin) is related to migration of leukocytes through the endothelial layer [30]. In addition to these adhesion molecules, some chemoattractive factors are important for the intimal migration of leukocytes adhered to the endothelium. Oxidized LDL and other factors expressed in the rising atheroma stimulate the production of MCP-1 (Monocyte Chemoattractant Protein-1) which is a great activator of monocyte chemotaxis [31]. The inflammatory cells widelyfound in the newly formed atheroma are macrophages and T-lymphocytes.Once transformed into foamy cells due to cytoplasmic lipids accumulation, these macrophages are found more frequently in atheroma, where cytokines and growth factors are produced.

The first pathologically relevant atherosclerotic lesion, the so called “lipid streak”, begins to form as foamy cells start to accumulate in the intimate layer. This process is still potentially reversible.

The foamy cells of the lipid streak acts as a promoterof the inflammatory process of the vascular wall, which synthesizes and releases cytokines, chemotactic factors and oxidizing radicals. This system of immune cells do not need antigenic stimuli to sustain itself [32]. TNF-α, IL-2, IL-6, IFN-γ, and other pro-inflammatory factors are widely produced by Th1 subpopulation, while Th2 lymphocytes, among other secreted factors, synthesize and release IL-10, the main anti-inflammatory cytokine. The role of acquired immunity, and in particular the T helper mediated one, could therefore orchestrate the inflammatory process triggered in the growing plaque. The cytotoxic T lymphocytes could mediate the release of lipolytic and proteolytic substances, oxidizing and inducing apoptosis of many cell types such as macrophages [33]. Activated SMCs (smooth muscle

cells) are also capable of secreting factors structurally similar to TGF-β and pro-calcifying moleculesas well ascalcium binding proteins. These molecules therefore, influence a common phenomenon in advanced atheromatous plaques: the mineralization. In growing atheroma, SMCs are not the only non-immune cells to migrate and replicate, but even endothelial cells may localize in the plaque and, under the stimulation of molecules such as VEGF and FGF (vascular-endothelial and fibroblast growth factor), can also organize themselves to form microscopic neovascular plexuses rich in adhesion molecules that promote cellmigration. These vascular structures also appear to be important for plaque progression, as demonstrated by the fact that anti-neoangiogenic factors can limit their development [34]. When plaque extends beyond remodeling positive capacity of the vessel involved, it begins to produce luminal stenosis, which progressively narrows the internal diameter of the artery and causes a reduction in blood flow. In the specific case of coronary arteries an imbalance between demand and request for oxygen in the myocardium, may lead to the development of a chronic ischemic heart disease. Activated monocytes are capable of produce cytokines (such as TNF-α and IL-6) that maintain and amplify the inflammatory cascade. The transductive pathway mediated by the transcription factor NF-κB plays a fundamental role in progression of atherosclerosis, and its activation leads to an increase in production of the main pro-inflammatory cytokines, including IL-6 and TNF-α [35,36,37]. In our study, we hypothesized that cytokines released by activated macrophages and cells localized in the vascular wall may play an important role in the formation of atherosclerotic lesions, triggering the upregulation of vasoactive substances and growth factors. In fact, previous studies [38,39] have pointed out that the inflammatory cell infiltrate is more expressed during the early phases of atheromas growth, suggesting that inflammatory cytokines are responsible for the initiation of formation of the atherosclerotic lesions. Neoangiogenesis is a typical feature found in more advanced plaques. Angiogenesis is physiologically regulated by VEGF and its inhibitors. Hypoxia is the major stimulus for the production and cellular release of VEGF. In atherosclerotic lesions there is an expression of VEGF caused by hypoxia and release of molecules by macrophages. Small vessels then form inside the plaque and this phenomenon makes the plaque more unstable, thus facilitating the macrophage recall and a possible internal bleeding. VEGF is expressed by endothelial cells, macrophages and smooth muscle cells [40,41]. The presence of VEGF in the carotid plaque correlates with the presence of a newly formed vascular component, smooth muscle cells and inflammation cells [40,42]. The correlation between the presence of VEGF in the plaque and structural alterations (breaking of the fibrous cap), greater positivity to CD34 (endothelial marker) and expression of VEGFR1 in the present cells has been clearly demonstrated. The evaluation of blood levels of VEGF may be a useful tool to identify atherosclerotic lesions rich in microvessels and therefore more unstable [43,44]. Monitoring of elements related to the neoformation of

atherosclerotic plaquesis the primary objective of numerous new studies which take into consideration the serious consequences that could be caused by the presence of an atherosclerotic plaque in the carotid district, which really represents a high risk condition for the development of cerebrovascular events.

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Table I. Expression levels (%) of TGF-β1, IL-1β, TNF-α, ICAM-1 in coronary arterial wall of pigs, and respective levels of statistical significance (t-test).

	Group 1 pigs 18–24 weeks old	**Group 2 pigs 24–30 weeks old**	**P-value**
TGF-β	28.20 ±6.28	29.10±4.15	p=0.7098
TNF-α	31.70 ±6.24	35.10±4.53	p=0.1802
ICAM-1	25.70±5.48	28.00±4.19	p=0.3057
IL-1β	34.70±4.50	38.20±3.80	p=0.0765

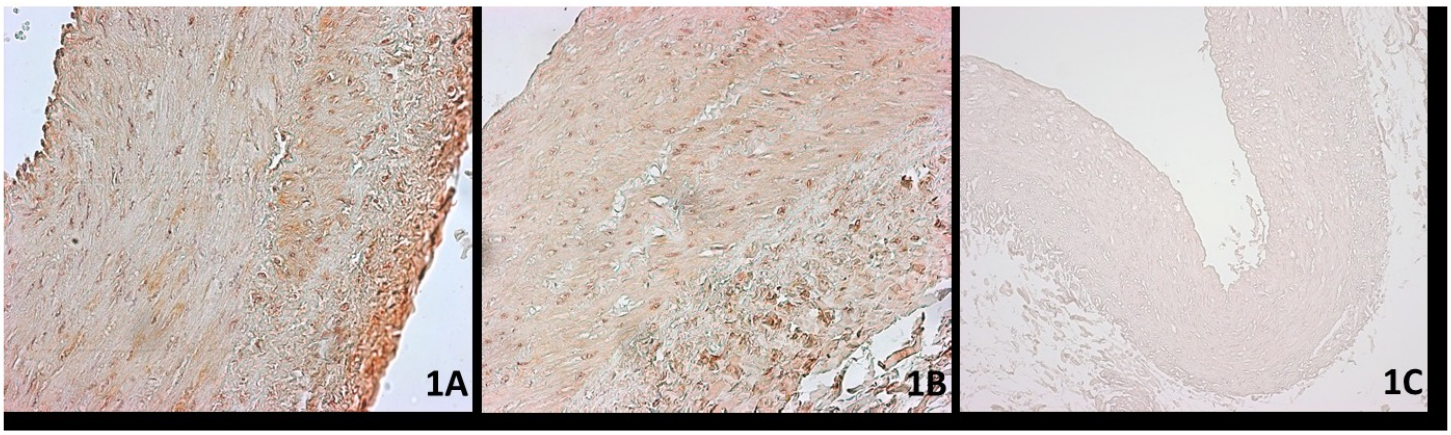
The results were considered as statistically significant when P-value<0.05

Figure 1. Immunohistochemical staining of porcine coronary arteries for IL-1b. Fig. (1A-1B) IL-1β was observedin the endothelial cells and in the nuclei of the smooth muscle cells of the media.No adventitial inflammation was observed, we did not observe any significant neointima formation in all the samples examined. TI (tunica intima), TM (tunica media), TA (tunica adventitia). Fig.1C negative controls, without antibody. (1A- x20; 1B-40x; 1C-10x).

Figure 2. Immunohistochemical staining of porcine coronary arteries for TGF-β1. Representative micrographs of vascular wall morphology are shown in Figures (2A-2B).TGF-β1 shows positivity of the endothelium and of the nuclei of the smooth muscle cells of the media,we did not observe any significant neointima formation in all the samples examined. TI (tunica intima), TM (tunica media), TA (tunica adventitia). Fig.2C negative controls, without antibody. (2A- x10; 2B- x40; 2C-20x).

Figure 3. Immunohistochemical staining of porcine coronary arteries for ICAM-1. Fig. (3A-3B) ICAM-1 shows positivity in the endothelium and in the nuclei of smooth muscle cells of the media. No adventitial inflammation was observed. TI (tunica intima), TM (tunica media), TA (tunica adventitia). Fig.3C negative controls, without antibody.(3A- x10; 3B-x40; 3C-40x).

Figure 4. Immunohistochemical staining of porcine coronary arteries for TNF-α Representative micrographs of vascular wall morphology are shown in Figures (4A-4B). TNF-α was expressed weak positivity in some nuclei of the smooth muscle cells of the media. TI (tunica intima), TM (tunica media), TA (tunica adventitia). Fig.4C negative controls, without antibody.(4A- x10; 4B-x40; 4C-40x).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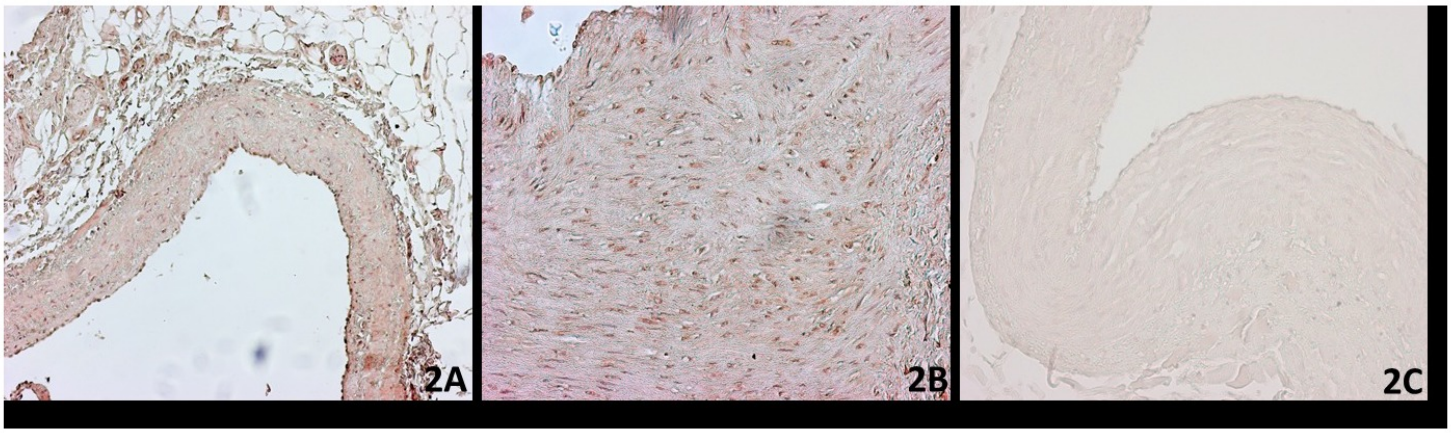
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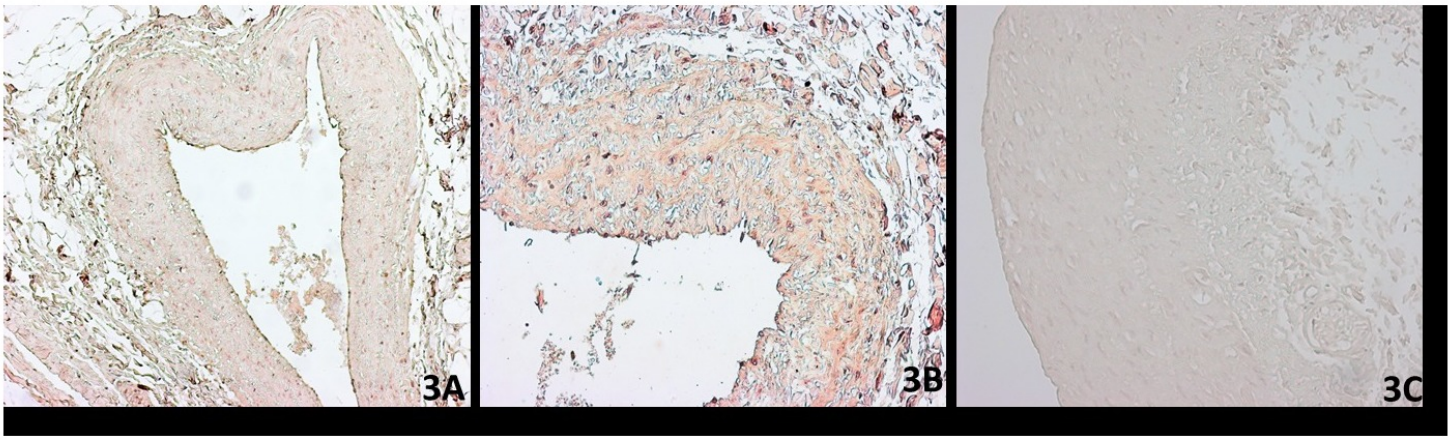
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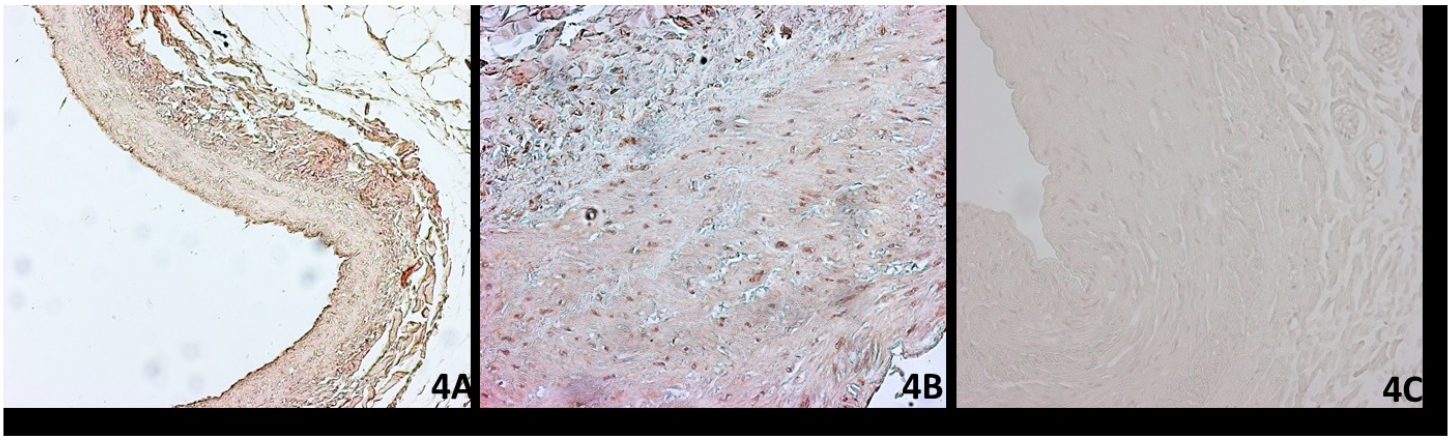
4A
4B
4C