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Presence of cardiomyocytes exhibiting Purkinje-type morphology and prominent connexin 45 immunoreactivity in the myocardial sleeves of cardiac veins

Szilvia Kugler, MD, Nándor Nagy, PhD, Gergely Rácz, MD, PhD, Anna-Mária Tőkés, PhD, Bence Dorogi, MD, Ágnes Nemeskéri, MD, PhD

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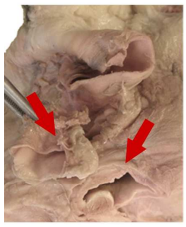
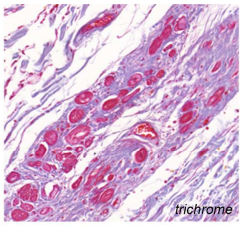
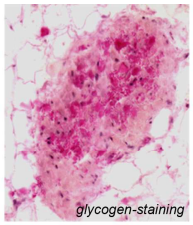


Myocardial sleeve of pulmonary veins (PV)



Purkinje-like cells *(red)* embedded in network of collagen fibers *(blue)* in PV

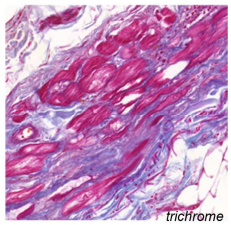


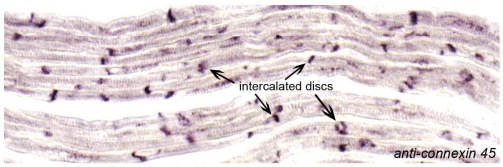
Strong glycogen staining of PV myocardium

Purkinje-like cells are also present

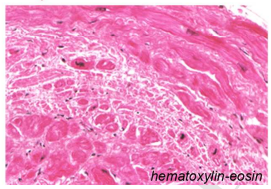
in the myocardial sleeve of human superior caval vein

and coronary sinus





Prominent connexin 45 immunoreactivity of PV myocardium



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Presence of cardiomyocytes exhibiting Purkinje-type morphology and prominent connexin 45 immunoreactivity in the myocardial sleeves of cardiac veins

Short title: Immunohistology of cardiac veins' myocardium

Szilvia Kugler MD[a], Nándor Nagy PhD[a], Gergely Rácz MD, PhD[b], Anna-Mária Tőkés PhD[c], Bence Dorogi MD[a], Ágnes Nemeskéri MD, PhD[a]

[a]Department of Anatomy, Histology and Embryology, Faculty of Medicine, Semmelweis University, H-1094 Budapest, Tűzoltó u. 58., Hungary

[b]1st Department of Pathology and Experimental Cancer Research, Faculty of Medicine, Semmelweis University, H-1085 Budapest, Üllői út 26., Hungary

[c]2nd Department of Pathology, Faculty of Medicine, Semmelweis University, H-1091 Budapest, Üllői út 93., Hungary

Correspondence to Ágnes Nemeskéri MD, PhD, Semmelweis University, H-1085 Budapest, Üllői út 26, Hungary; e-mail address: nemeskeri.agnes@med.semmelweis-univ.hu; telephone number: 0036 1 215 69 20

Conflict of interest: none.

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Abstract

Background Pulmonary vein (PV) myocardium is a known source of atrial fibrillation. A debated question is whether myocardial extensions into caval veins and coronary sinus (CS) have similar properties. No studies have documented specific pacemaker and/or conducting properties for the human extracardiac myocardium.

Objective The aim was to characterize the histology and immunohistochemical features of myocardial sleeves in the wall of cardiac veins.

Methods Sections of 32 human hearts were investigated. Specimens of PVs, superior caval vein (SVC), CS, sinoatrial and atrioventricular nodes and left ventricle were stained with Best's Carmine for selective staining of intracellular glycogen. Anti-connexin (Cx)-45 and Cx43 specific antibodies were used to determine the conduction properties of extracardiac myocardium.

Results Myocardial sleeve was found in the wall of PVs of 15/16 hearts, 21/22 SVCs and 8/8 CSs. Bundles of glycogen positive cardiomyocytes exhibiting pale cytoplasm and peripheral myofibrils were observed in the venous sleeves. Strong Cx45 and weak Cx43 labeling was detected in the extracardiac myocardium. Similar staining pattern was observed at the pacemaker and conduction system, while ventricular myocardium exhibited prominent Cx43 and no Cx45 immunoreactivity.

Conclusion Myocardial fibers of PVs, SVC and CS exhibit similar morphology to that of Purkinje fibers and are enriched in glycogen. We provide data for the first time, on the prominent positive staining for Cx45 in the extracardiac myocardium, indicating its potential pacemaker and/or conducting nature.

Keywords: pulmonary vein; caval vein; coronary sinus; cardiac muscle sleeve; Purkinje-type morphology; glycogen; connexin 45; immunohistochemistry

Introduction

Myocardial sleeve of pulmonary veins (PV) play a critical role in the mechanism of atrial fibrillation (AF). Macroscopic features of these areas were described previously.[1-3] During the last decade, growing attention has prompted to the microscopic properties of the extracardiac myocardial sleeves.[2,4-6] In the wall of human PVs, Perez-Lugones et al.[7] documented the presence of cardiomyocytes exhibiting ultrastructural morphology of P-cells and Purkinje fibers (PFs). Although accepted that atrial tachyarrhythmias are frequently triggered from caval veins and coronary sinus (CS)[8-12], limited data has been published about macroscopic[3] and microscopic morphology[13,14] of the myocardial sleeves of these regions. Moreover, there is a general lack of research in the immunohistochemical characterization of caval and CS myocardial sleeves.

Immunohistochemical markers to distinguish working myocardium, pacemaker or conducting cells are established. Besides several determinants of conduction in the heart, such as HCN4 and HNK-1, connexin (Cx) isoforms, of which gap junction channels are comprised are also characteristic proteins of cardiac pacemaker tissues. Cx40, Cx43 and Cx45 are found differentially expressed in cardiomyocytes at various sites, which determines the characteristics in conduction velocity.[15] Cx43 is present throughout the working myocardium, whereas Cx40 is confined to the atrial myocardium and the ventricular conduction system. Cx45 is predominantly expressed in the impulse generating and conduction system, while it is present in substantially lower amounts in the working myocardium.[16,17]

Methods

Human tissues

Thirty two adult human hearts were removed from cadavers at 12-72 hours postmortem age kept at 1-5 ^{o}C until fixation. The ages of deceased individuals ranged

between 60 and 81 years. Their medical histories were unknown. Prior to death donors gave written consent for the use of their bodies for education and research {Willed (Whole) Body Program - WWBP}. The work has been ethically approved by the Regional and Institutional Committee of Science and Research Ethics, Semmelweis University (Research Ethics committee approval 122/2016).

Due to technical reasons, the heart could be excised together with PVs only in 16/33 and with SVC in 22/33 subjects. The excision was extended into the lung hilum in the case of PVs, above the level of azygos vein regarding SVC and as far as the orifice of great cardiac vein in the case of CS. The veins were then separated from the atria at the level of their ostia and were cut transversely. CSs of 8/33 subjects were suitable for further tissue processing. Tissue samples were obtained from the sinoatrial and atrioventricular nodes, the atria, anterior wall of the left ventricle and the interventricular septum. Specimens were either fixed in 4% formaldehyde, or in 70% ethanol or in methanol. After dehydration in graded concentrations of alcohol, tissue samples were embedded in paraffin and 3-4 μm sections were made.

Tissue processing for histology

For general histology, paraffin sections were stained with hematoxylin and eosin (HE) or trichrome. Intracellular glycogen was demonstrated by Best's Carmine stain, which is a specific stain for glycogen content. Best's Carmine stain was performed as described.

Immunohistochemistry

For Cx45 immunohistochemistry, specimens were fixed in ethanol/methanol and embedded in paraffin. After deparaffinization and rehydration through graded alcohols, the slides were washed three times in phosphate-buffered saline (PBS). Heat-induced antigen retrieval method was applied using Tris-based (Target Retrieval Solution pH-9; Dako) or citrate-based (Sigma; H-3300) antigen unmasking solution, respectively. For Cx43 immunohistochemistry, frozen sections were prepared. Specimens were embedded in

Cryomatrix (Shandon), frozen in liquid nitrogen and stored in deep freezer (-80 ^{o}C). 10 μm thin cryosections were mounted on poly-L-lysine coated slides, fixed in cold (+4 ^{o}C) acetone for 10 minutes and air dried. Before immunostaining the slides were rehydrated in PBS and permeabilization with 0,3% Triton X-100 was carried out for 40 minutes.

Cx45 and Cx43 immunostainings were prepared as follows. Protein blocking was carried out for 15 minutes with 1% BSA in PBS. It was followed by overnight incubation at 4 ^{o}C with primary antibodies: Cx45 was detected with a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.; sc-25716; dilution 1:100), Cx43 was detected with a goat polyclonal antibody (Santa Cruz Biotechnology, Inc.; sc-6560; dilution 1:50). Secondary antibodies, which included biotinylated goat anti-rabbit IgG and biotinylated horse anti-goat IgG (Vector Labs) were used and followed by endogenous peroxidase activity quenching step using 3% hydrogen peroxide (Sigma) in PBS. After formation of the avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit; Vector), the binding sites of the primary antibodies were visualized by 4-chloro-1-naphthol (Sigma).

The sections were covered by aqueous Poly/Mount (Polyscience, Inc., Warrington, PA) and examined by Zeiss Axiophot photomicroscope. An automated 3D-Histotech whole slide imaging system was used to image the sections.

Results

Myocardial sleeve of the pulmonary veins

Extensions of left atrial myocardium could be observed in the PVs of 15/16 (94%) hearts and formed bundles displaying various course (Fig. 1A,B). Bundles of large cardiac cells (median diameter 18,1 (IQR: 16,5 – 19,7) μm) resembling of PFs due to their lightly stained cytoplasm and peripheral myofibrils were detected in the PVs of 14 hearts. Among these cardiomyocytes, dense network of fine collagen bundles was present (Fig. 1C,D). Best's

Carmine staining confirmed that PV myocardium was enriched in cardiomyocytes containing abundant glycogen (Fig. 1E). Intense Cx45 labeling was observed in the myocardial sleeve of PVs. Connexins were clustered in intercalated discs (Fig. 1F).

Myocardial sleeve of the superior caval vein

Myocardial sleeve composed of fibers displaying mainly spiral course was found around 21 of 22 (95%) SVCs (Fig. 2A,B). In one case, some groups of myocardial fibers were present at the root of the azygos vein but no cardiac cells were found in the portion distal to this point. Bundles of Purkinje-like cardiomyocytes (median diameter 29,4 (IQR: 27,9 – 32,5) μm) embedded in connective tissue were identified in 20 SVCs (Fig. 2C). Similar to PV abundant intracellular glycogen content was found in the SVC myocardium (Fig. 2D). We observed intense Cx45 positivity with a similar pattern as we described in Fig. 1F (Fig. 2E). No Cx43 staining in the sinoatrial node, sparse labeling in the vicinity of sinoatrial node (mixed population of atrial and pacemaker cells) and marked positivity were detected in the atrial working myocardium (data not shown).

Myocardial sleeve of the coronary sinus

Cardiac muscle was present in 8 of 8 (100%) CS specimens (Fig. 3A). The course of cardiomyocyte-bundles was spiral closer to the venous lumen and predominantly longitudinal at the outer circumference (Fig. 3B). Purkinje-like myocardial fibers (median diameter 22,7 (IQR: 20,7 – 25,5) μm) embedded in network of collagen fibers were identified in 7 CSs (Fig. 3C,D). Immunostaining revealed that Cx45 labelings are as prominent in intercalated discs as were described in PV and SVC (Fig. 3E). Cx43 labeling could barely be observed in the myocardial sleeve of CS.

Histology of the working myocardium and conduction system of the heart

The ventricular conducting cells were rich in glycogen contrary to the ventricular working myocardium (Fig. 4A,B,C). As compared to the extracardiac myocardium, no Cx45

signal could be detected in the working myocardium, while conducting cells proved to be strongly positive (Fig. 4D). Weak Cx45 immunoreactivity was present in the atria. Cx43 label was marked throughout the ventricular working myocardium (Fig. 4E). At the region of sinoatrial node prominent Cx45 immunoreactivity was detected. Cx45 was found to be present at the atrioventricular nodal region and in the atrioventricular bundle as well.

Discussion

Characteristics of myocardial extensions around pulmonary veins

It has been demonstrated that left atrial myocardium extends into the wall of PVs in a 68-100% proportion.[2,4-6] No specialized cells were observed except Perez-Lugones et al.[7] who analyzed electron microscopic images of human PV myocardium and reported the presence of P cells and PFs. Nguyen et al.[18] found PAS-positive cells, further supporting specialized characteristics of PVs. In the current study bundles of cardiomyocytes displaying the characteristic features of PFs were identified in almost all PV, SVC and CS samples. These cells contained high amount of glycogen in their cytoplasm and they were found to be embedded in a dense network of fine collagen fibers.

Potential arrhythmogenic role of caval veins and coronary sinus

Increasing interest has been recently devoted to non-PV ectopic beats that proved to be responsible for 20% - 32% of all AF cases.[11,12] Among patients with non-PV-initiating AF, SVC triggers were found in about 40%.[9,11,12] In the wall of SVC, myocardial sleeve was detected in 76% - 78% of all cases.[3,14] CS area were recognized as sites of tachyarrhythmias in 1% - 17%[11,12] of all non-PV ectopies. Two studies noted the presence of myocardium in all CSs examined[19,20] while DeSimone et al.[3] reported that only 7% of the CSs contained myocardial extensions from the right atrium. To the best of our knowledge, the present study demonstrates for the first time that cells displaying PF morphology are present in human SVC

and CS myocardium. The question arises whether this may indicate possible arrhythmogenicity of these regions. We intend to investigate hearts removed from deceased patients who possessed evidenced extracardiac loci of atrial arrhythmias to provide data for the clarification of this issue.

Immunohistochemical characterization of extracardiac myocardial sleeves

In order to characterize the immunophenotype of the extracardiac myocardium, we carried out immunostainings. Until now, positivity for HNK-1, which is an antigen to the developing conduction system[21], and reactivity for the cardiac pacemaker antigen HCN4 were detected by human studies.[18,22]

According to previous data Cx45 was detected at very low levels in atrial and ventricular working myocardium,[16] whereas distinct positive signal was found at the atrioventricular node of human adults.[17] Therefore Cx45 seems to be a specific marker of the conduction system. During the current study, strong staining for Cx45 was observed throughout the impulse generating and conduction system of the heart, whereas almost no immunopositivity could be detected in the working myocardium.

Data on the connexin expression patterns of cardiac veins are based on animal researches. Differences regarding distinct species were published. In canine SVC, the presence of all cardiac connexins including Cx45 were reported, with distinct areas characterized by abundance of Cx43 in the center and diffuse Cx40 signals in the periphery. Such areas of atypical connexin expression were mainly present in the proximal portion of the SVC, usually in the outer circumference of the myocardial sleeve.[23] Both Cx40 and Cx43 were observed in isolated cardiac cells from canine great veins, with a higher amount of Cx43 in the SVC than in the PVs. The absence of Cx45 signal was presumably caused by paraformaldehyde fixation or injury of the cell membrane.[24] In rat, a nodal-like tissue looping around the junction of right atrium and SVC was reported. From the junction lightly stained

cells extended both next to the crista terminalis and the interatrial groove. While nodal-like cells proved to be strongly positive for Cx45 and negative for Cx43, atrial walls and PV myocardium exhibited intense Cx43 but no Cx45 immunostaining in rat.[25]

The current study documents for the first time the presence of Cx45 positive myocardial fibers in the wall of human PVs, SVC and CS. Based on the difference between Cx45 immunopositivity of the working myocardium and the pacemaker and/or conducting structures, our findings regarding the prominent Cx45 staining of the extracardiac myocardial fibers might provide some support for the presumed specialized nature of these areas. Since Cx43 labeling was weak in the myocardial sleeves, it can be hypothesized that extracardiac myocardium in PVs, SVC and CS contains predominantly cardiomyocytes with pacemaker and/or conducting nature.

Limitation

Immunohistochemistry is not an adequate method for reporting amounts of protein expression. Therefore application of quantitative western blot analysis which is suitable for determining relative abundance of distinct proteins would add weight to our observations.

Conclusions

1. Presence of Purkinje-like cardiomyocytes exhibiting strong glycogen positivity was documented in the myocardial sleeves of human PVs, SVC and CS.

2. This research is the first to demonstrate pronounced Cx45 positivity of the extracardiac myocardium which might provide some support for the presumed arrhythmogenicity of the myocardial sleeves ensheathing PVs, caval veins and CS.

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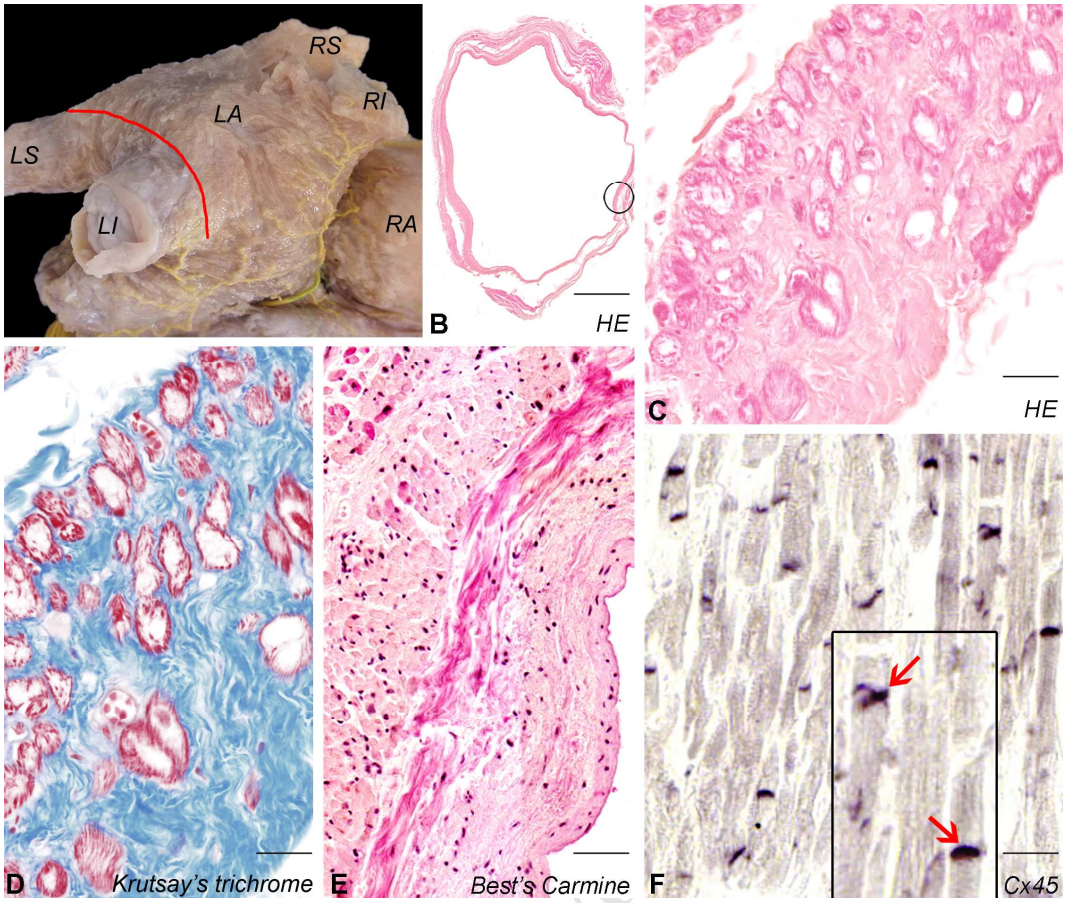

Figure 1. Histology of the PV myocardium. Myocardial extensions into the wall of PVs (*LS, LI, RS, RI*). *LA* and *RA*= left and right atria. Circumflex artery is filled up by yellow resin mixture. Red line shows the area which was cut out for histology (A). Transverse section of a PV with myocardial sleeve (B). Large cardiomyocytes with lightly staining cytoplasm at the area signed by circle at picture B (C). Trichrome staining shows that myocardial fibers (red) are isolated by fibrous tissue (blue) (D). A bundle of myocardial cells containing much glycogen (E). Prominent Cx45 positivity in intercalated discs (inset, arrows) (F). Scale bar: 5000 μm (B); 40 μm (C); 30 μm (D); 70 μm (E); 30 μm (F); 20 μm (F-inset).

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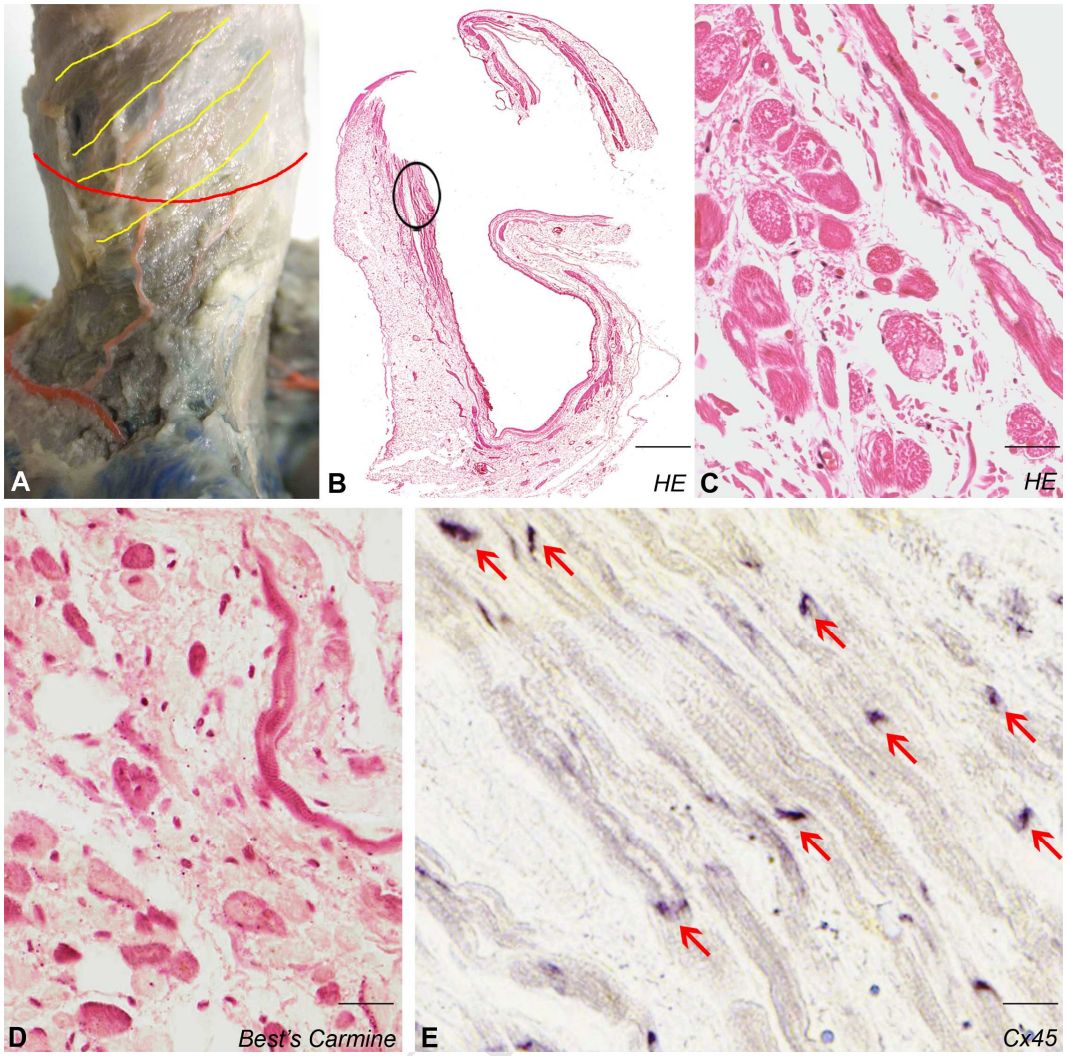

Figure 2. Histology of the SVC myocardium. Cardiomyocyte bundles with spiral course (yellow lines). Sinoatrial nodal artery is filled up by red resin mixture. Red line shows the area which was cut out for histology (A). Transverse section of SVC with myocardial sleeve (B). Purkinje-like myocardial cells at the area signed by circle at picture B (C). Glycogen containing cardiomyocytes (D). In intercalated discs, Cx45 immunoreactivity is prominent (E, arrows). Scale bar: 1750 μm (B); 30 μm (C); 30 μm (D); 20 μm (E).

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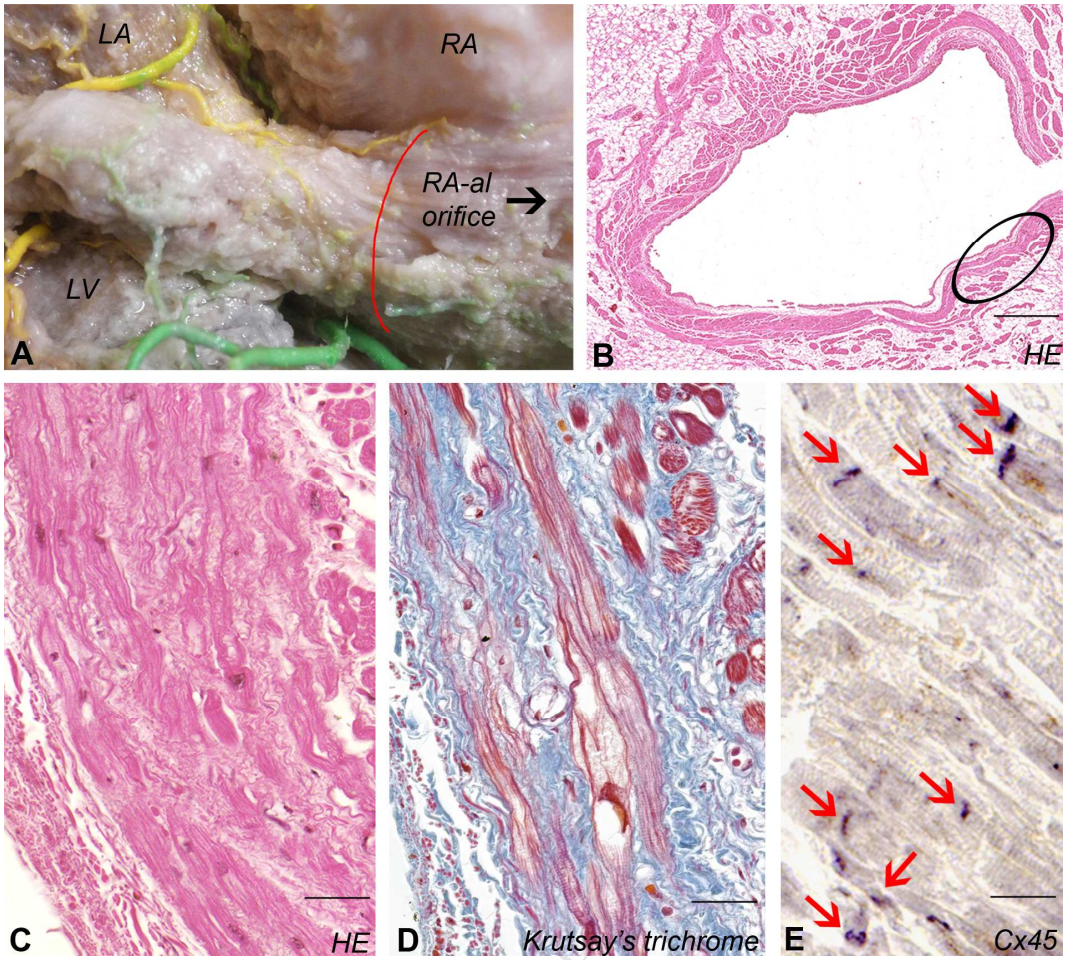

372 **Figure 3. Histology of the CS myocardium**. Myocardial sleeve around the wall of CS. *RA*=right
373 atrium, *LA* and *LV*= left atrium and ventricle. Right coronary artery (green) and circumflex artery
374 (yellow) are filled up by synthetic resin. Red line shows the area which was cut out for histology
375 (A). Cross section of the CS orifice (B). Bundles of Purkinje-like cardiomyocytes run around the
376 lumen at the area signed by circle at picture B (C). Trichrome staining shows that myocardial
377 fibers having lightly staining cytoplasm (red) are separated by connective tissue (blue) (D). Cx45
378 immunoreactivity is prominent at intercalated discs (E, arrows). Scale bar: 900 μm (B); 45 μm
379 (C); 40 μm (D); 30 μm (E).
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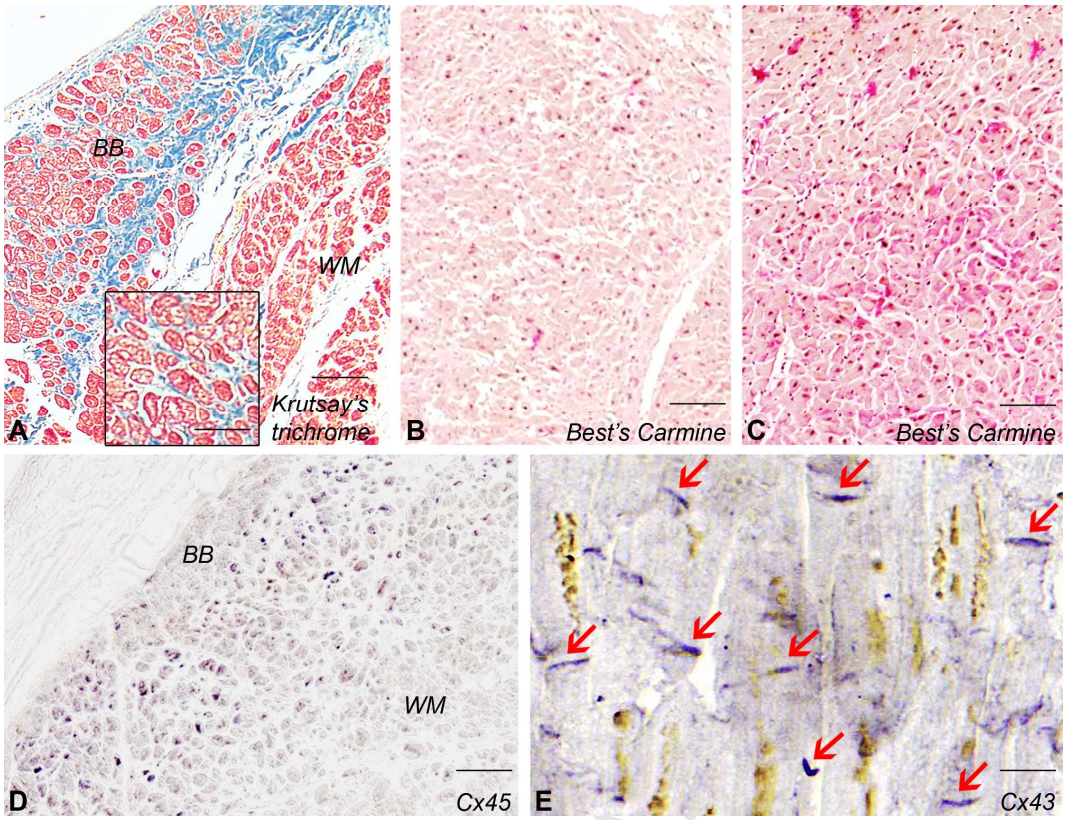

384 **Figure 4. Histology of the left ventricular myocardium.** Trichrome staining of the working
385 myocardium (*WM*) and a bundle branch (*BB*, inset) composed of cardiomyocytes with pale
386 cytoplasm (A). WM shows almost no sign with glycogen specific Best's Carmine staining (B),
387 while much glycogen is recognized at the BB (C). Cx45 immunoreactivity is prominent at the *BB*
388 but barely detectable in the *WM* (D). At the WM, marked positivity for Cx43 is detectable in
389 intercalated discs (arrows). Yellow-brown intracellular granules in the vicinity of nuclei are
390 lipofuscin pigments (E). Scale bar: 80 μm (A); 40 μm (A-inset); 160 μm (B); 100 μm (C); 90 μm
391 (D); 12 μm (E).

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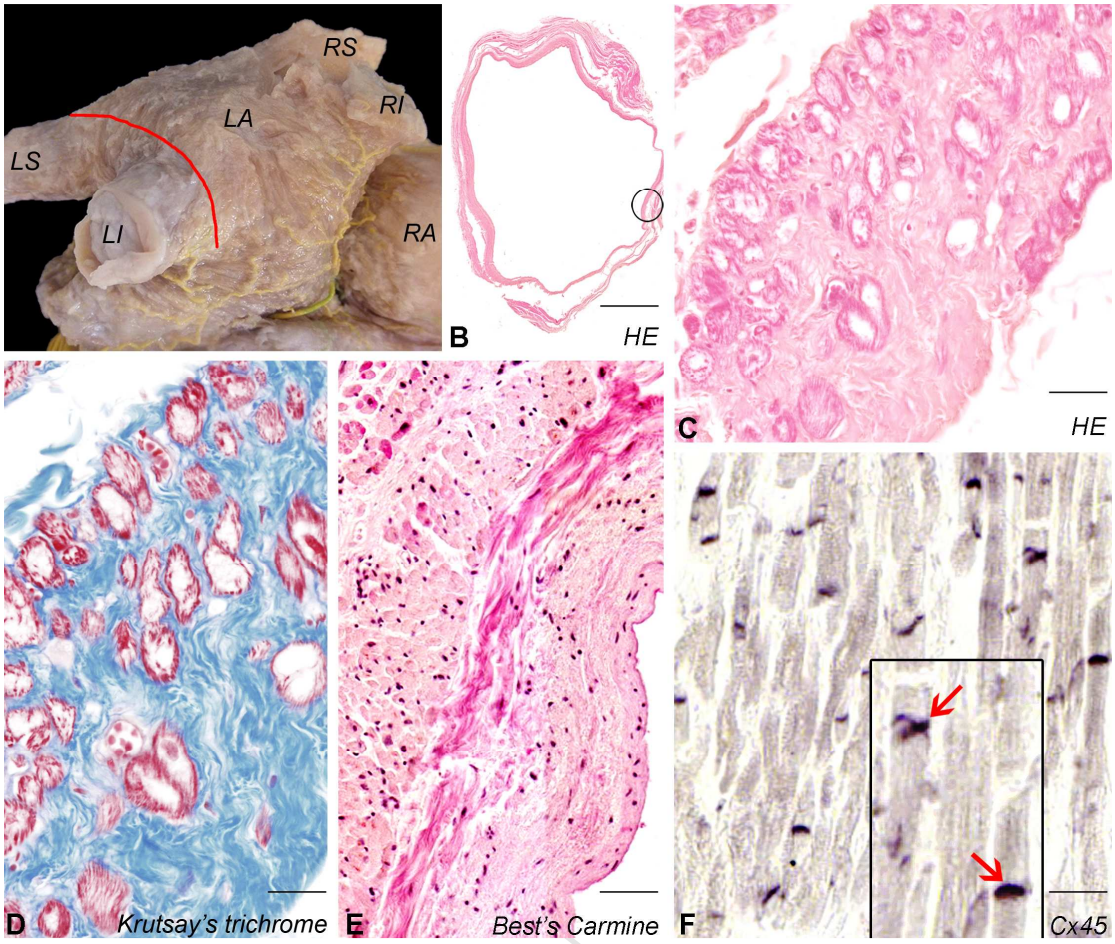

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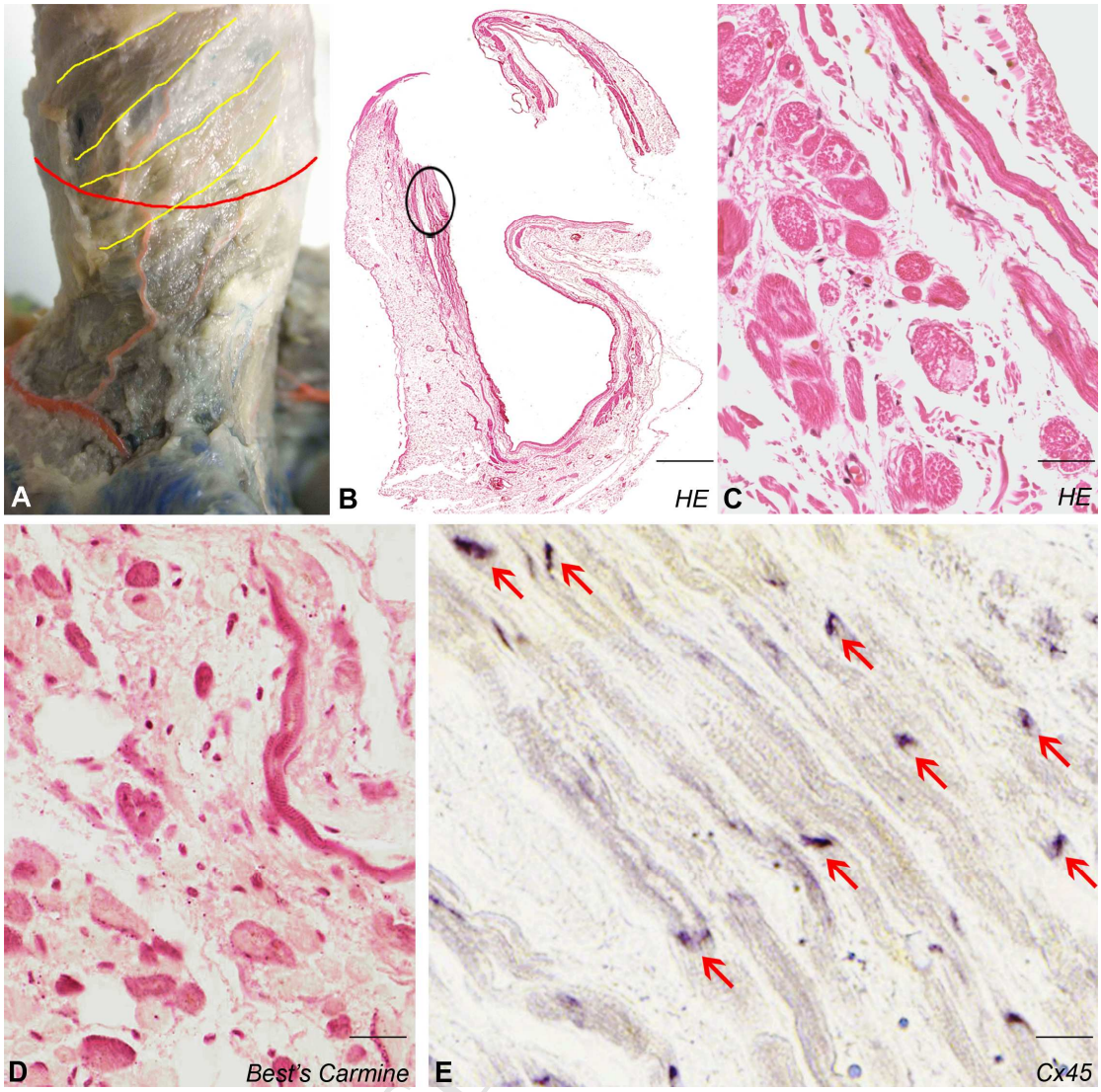

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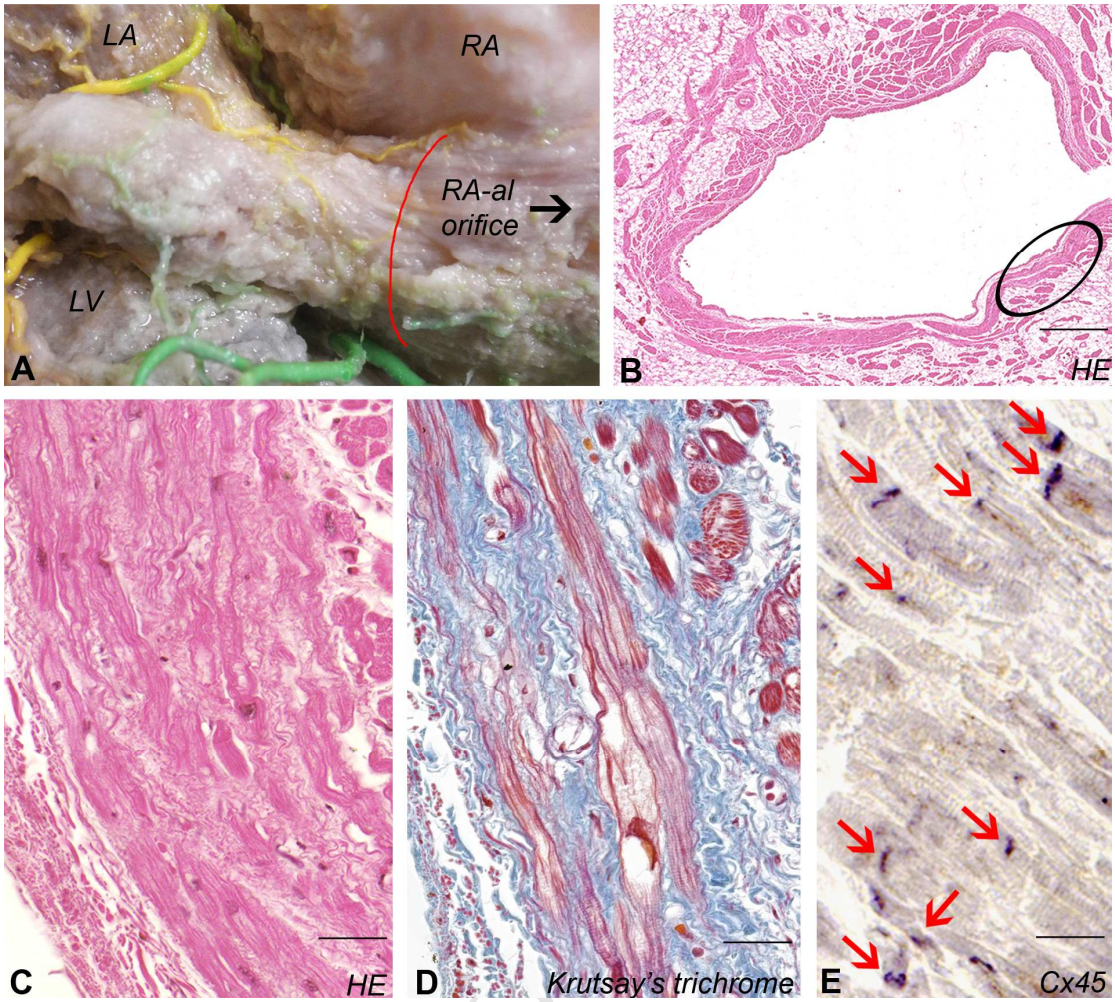

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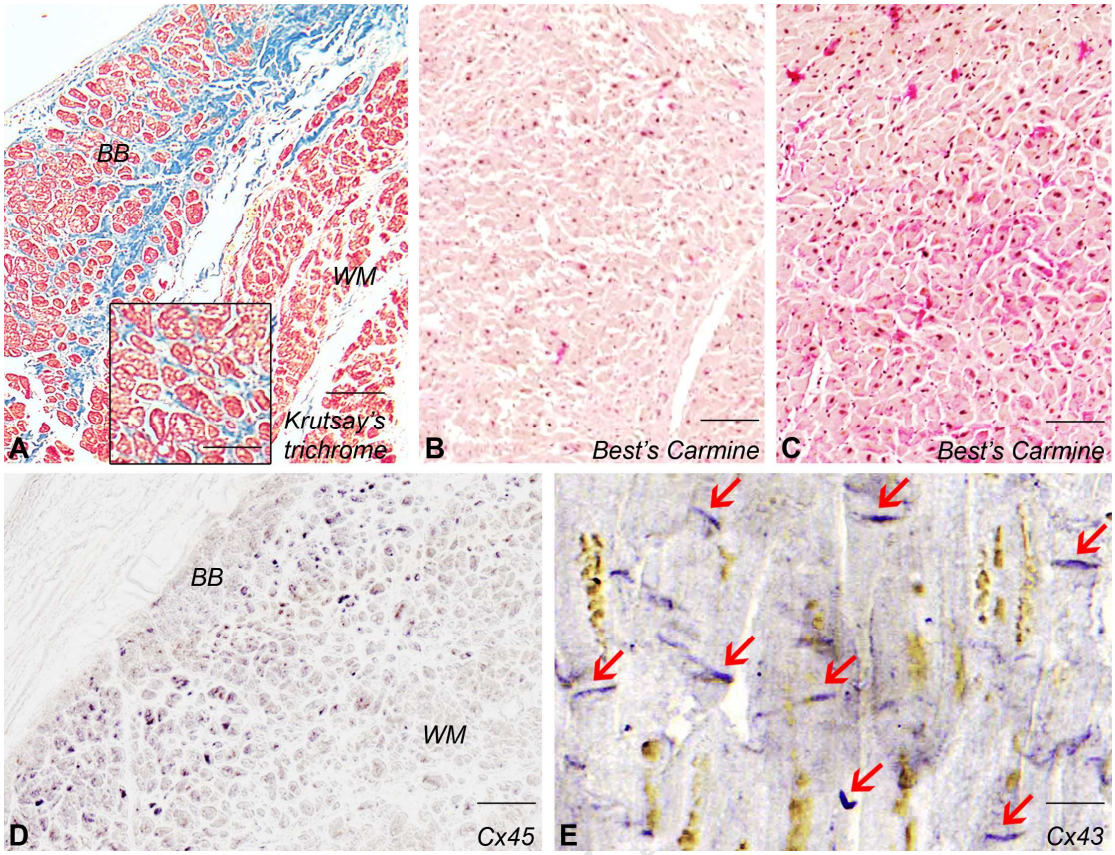

ACCEPTED MANUSCRIPT