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DOI:

[10.1016/j.hrthm.2019.07.033](https://doi.org/10.1016/j.hrthm.2019.07.033)

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Document Version

Peer reviewed version

Citation for published version (Harvard):

De Almeida, MC, Stephenson, RS, Anderson, RH, Benvenuti, LA, Loukas, M & Aiello, VD 2020, 'Human subpulmonary infundibulum has an endocardial network of specialized conducting cardiomyocytes', *Heart Rhythm*, vol. 17, no. 1, pp. 123-130. <https://doi.org/10.1016/j.hrthm.2019.07.033>

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The human subpulmonary infundibulum has an endocardial network of specialized conducting cardiomyocytes. Short title: Human subpulmonary infundibular network

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PII: S1547-5271(19)30719-2

DOI: <https://doi.org/10.1016/j.hrthm.2019.07.033>

Reference: HRTM 8101

To appear in: *Heart Rhythm*

Received Date: 19 June 2019

Please cite this article as: De Almeida MC, Stephenson RS, Anderson RH, Benvenuti LA, Loukas M, Aiello VD, The human subpulmonary infundibulum has an endocardial network of specialized conducting cardiomyocytes. Short title: Human subpulmonary infundibular network *Heart Rhythm* (2019), doi: <https://doi.org/10.1016/j.hrthm.2019.07.033>.

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The human subpulmonary infundibulum has an endocardial network of specialized conducting cardiomyocytes.

Short title: Human subpulmonary infundibular network

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Conflict of interest statement: the authors have no conflicts of interest.

Total of words: 3727

Abstract

Background: The right ventricular outflow tract is the most common source of ventricular arrhythmias in non-structural heart disease. Most of these arrhythmias are of endocardial origin, but their morphological substrate is mostly unknown.

Objective: To identify potential morphological substrates for such arrhythmias originating within the right ventricular outflow tract.

Methods: We examined three adult human hearts, which had been fixed in 4% formaldehyde. Of the hearts, two were obtained subsequent to necropsies. In these hearts, the base of the anterior papillary muscle of the tricuspid valve was removed at the site of its fusion with the moderator band. The block of myocardium thus removed was submitted to routine histological processing and sectioned at 5 micrometer thickness. The free-standing sub pulmonary infundibulum was also removed as a series of macroscopic preparations, which were then similarly sectioned in their short axis at 5 micrometer thickness. The third heart was assessed using micro-computed tomography after it had been stained with 7.5% I2KI contrast agent for 14 days, with the contrast agent refreshed at the seventh day.

Results: We identified, and tracked, specialized conducting cardiomyocytes from the base of anterior papillary muscle to the supraventricular crest and

subpulmonary infundibulum, using histology in two, and microcomputed tomography in the other. Transitional cells were also found at these sites.

Conclusion: The existence of such specialized cardiomyocytes within the infundibulum is of obvious clinical significance, since they could be the morphological substrate for the arrhythmias known to originate from these sites.

Keywords: Human pulmonary subinfundibulum, Heart, Specialized Conducting Cardiomyocytes Network, Arrhythmia, Transitional cells, Supra Ventricular Crest, Right Ventricle.

Introduction

Despite intensive research in the anatomical sciences for the past two centuries, some structures of the human body still remain controversial, or ill-described. One of these topics is the extent of the sub-endocardial specialized cardiomyocytes. Standard accounts, such as provided by Tawara, and Aschoff and Monckeberg, describe the presence of these specialized cardiomyocytes exclusively beneath the endocardium, with the cells isolated within connective tissue sleeves. The only other groups of cells found scattered within the endocardium are smooth muscle cells. Such endocardial ramifications of the ventricular conduction system in the

human heart are presumed to be a source of arrhythmias¹⁻³. Their three-dimensional distribution, however, has yet to be systematically described. Changes in the architecture of the ramifications, furthermore, may be the substrate for variable conduction velocities in different parts of the heart^{2,4}. The macroscopic pattern of the ramifications has been demonstrated in hearts from ungulates^{5,6}. In these species, rich networks were demonstrated in the infundibulum, and on the supraventricular crest. Thus far, comparable visualization has not been achieved in humans,^{7,8} although electrophysiological investigations have shown the existence of potential arrhythmic substrates in the subpulmonary infundibulum.⁹⁻¹⁵ It is generally agreed that cardiac structure is relatively well-preserved among mammalian species.¹⁶⁻²⁰ It is reasonable to assume, therefore, that comparable ramifications of the bundle branches will be present in the outflow tracts of human hearts. Our aim in this study, therefore, was to examine grossly and microscopically the ramifications of the right bundle branch within the free-standing subpulmonary infundibulum.

Methodology

We examined three adult human hearts, which had been fixed in 4% formaldehyde. Of the hearts, two were obtained subsequent to necropsies performed at the Heart Institute (InCor) of Sao Paulo University School of Medicine. The necropsies were

done after obtaining an appropriate agreement form, which granted permission for the use of organs and tissues in scientific research. The first heart was normal, obtained from a 58-year-old woman who died from acute idiopathic interstitial pneumonia. The other heart showed severe biatrial dilation and mild left ventricular hypertrophy and fibrosis. This heart was obtained from a 40-year-old woman with chronic rheumatic heart disease who had a calcified mitral valvar prosthesis, and who died subsequent to an acute myocardial infarction. The third heart had previously been described in the study of Stephenson and colleagues, and was assessed using micro-computed tomography⁸. In the two necropsied hearts, as shown in Figure 1, the base of the anterior papillary muscle of the tricuspid valve was removed at the site of its fusion with the moderator band. The block of myocardium thus removed was then submitted to routine histological processing and sectioned at 5 micrometer thickness. The free-standing sub pulmonary infundibulum was then removed as a series of macroscopic preparations, which were then similarly sectioned in their short axis at 5 micrometer thickness (Fig. 2 A, B). The supraventricular crest was also removed and cut into macroscopic blocks perpendicular to its long axis, with the blocks again being sectioned at 5 micrometers thickness. All the sections were stained with hematoxylin and eosin, with reticulum and trichrome staining being performed in selected samples. A total of 25 histological sections were prepared for each heart.

Histological analysis

We distinguished specialized conducting cardiomyocytes from working cardiomyocytes using the following criteria:

- their exclusive endocardial localization.
- their isolation from the subjacent working myocardium by endocardial fibrous tissue, apart from focal sites where it proved possible to identify cells interpreted as transitional cells^{16,17}. The latter were defined as cells that connected endocardial aggregates of specialized conducting cardiomyocytes to myocardial working cardiomyocytes. These cells are, in general, slenderer than either the specialized endocardial conducting cardiomyocytes or the working cardiomyocytes.
- their individual staining characteristics, on the basis that they possessed fewer myofibrils than adjacent working cardiomyocytes, and showed a more evenly vacuolated cytoplasm around their nucleus.^{17,21}
- their size, in general, being marginally larger than the working cardiomyocytes.^{17,21}
- the finding, when grouped together, of less interstitial space, and fewer capillaries.²²

Micro-computed tomographic imaging

The third heart was obtained and prepared at the University of Minnesota, United States of America. It was recovered from an organ donor, whose heart was considered unsuitable for a transplant. The heart was arrested by cardioplegia, recovered, and delivered to the University of Minnesota within 12 hours via LifeSource (Minneapolis, MN). All appropriate consents were obtained for donation for this heart for research (HH074, for additional information <http://www.vhlab.umn.edu/atlas/histories/>). The heart was flushed with saline solution to remove any residing blood, and perfusion-fixed with 10% formalin for 48 hours, as previously described^{8,23,24}. The heart was then transported to the University of Manchester. A Materials Transfer Agreement was arranged, and shipping methods followed the guidelines of the Environmental Health & Safety Department of the University of Manchester. Prior to micro-computed tomographic scanning, the heart was stained with 7.5% I₂KI contrast agent for 14 days, with the contrast agent refreshed at the seventh day.^{8,23,24} To ensure the heart chambers were in a blood-filled state, the heart was filled with warm agarose solution, which becomes a firm low X-ray attenuating gel at room temperature. Scanning was carried out using the Nikon Metris XTEK 320 kV Custom Bay at the Manchester X-Ray Imaging Facility, University of Manchester, as previously described.^{8,23,24} The samples were cleared of excess contrast medium by washing in distilled water, and then immobilized in a plastic holder to prevent movement

during the imaging process. Scans were acquired with X-ray energies ranging from 85–160 kV. Scans were performed through 360 degrees, and data was collected over 3142 projections with two-frame averaging. A tungsten target was used for all scans, with a 1 mm aluminum filter. Total scan times were approximately 50 minutes. Data was reconstructed using filtered back-projection, resulting in tomographic image data with an isotropic voxel size of $73 \times 73 \times 73 \mu\text{m}^3$. Data sets were initially viewed, cropped, and manipulated using ImageJ 1.45i (<http://rsbweb.nih.gov/ij/>). Three-dimensional reconstructions of the regions of interest were then created in Amira 5.33, using the volume rendering and segmentation techniques previously described.^{8,23,25} Here, we present volume renderings of the intact human heart, in which segmentations of the cardiac conduction system are overlaid.

Results

Cells fulfilling the criteria of specialized conducting cardiomyocytes (Figs 3 and 4 A-D) were abundant at the base of the anterior papillary muscle, and also within adjacent false tendinous cords (Figs 4B). Specialized cardiomyocytes were similarly abundant in the proximal half of the sub-pulmonary infundibulum (Fig 5), where they formed aggregates within the endocardium (Figs. 3 A, C). The specialized cardiomyocytes could be tracked distally from their origin from the right bundle branch. No direct ramifications into the subpulmonary infundibulum

were observed from the proximal part of the bundle branch in the region of the medial papillary muscle. Comparable aggregates could also be identified throughout the circumference of the free-standing infundibular sleeve (Figure 5). These ramification also took their origin from the right bundle branch at the level of the anterior papillary muscle, rather than at the level of the medial papillary muscle. Aggregates of specialized cardiomyocytes were also found within the distal infundibulum, but in fewer numbers. Of significance, no specialized cardiomyocytes were observed in the myocardial crescents found at the bases of the valvar sinuses distal to the hinges of the leaflets of the pulmonary valve. Small aggregated collections of specialized cardiomyocytes were found in the endocardium of the supraventricular crest (Fig 4C). At several points, we identified connections between some of the cells within the endocardial aggregates and the adjacent working cardiomyocytes (Figs 5 and 6A- D). These transitional cells were, in general, thinner than the specialized conducting cardiomyocytes or the working cardiomyocytes (Fig 6 A-D). Within the free-standing subpulmonary infundibular sleeve, in a belt approximately 1cm below the base of attachment of the pulmonary leaflets, we identified additional isolated cells with the general cytological characteristics of specialized conducting cardiomyocytes (Fig 7 A-D). Due to the insufficient spatial resolution, the criteria used to distinguish the specialized conducting cardiomyocytes in the histological sections could not be

applied to the micro-computed tomographic data. We have shown previously, nonetheless, that cells with low pixel values, that is with low X-ray attenuation when compared to the working myocardium, can correspond to specialized conducting cardiomyocytes. In this regard, the findings then corresponded with the histological data. We found an abundance of specialized cells at the base of the anterior papillary muscle, and also within adjacent false tendinous cords (Figs 1 and 8). As already emphasized, we were able to track these insulated specialised cardiomyocytes distally from their origin at the base of the anterior papillary muscle, with no direct ramifications found at the level of the medial papillary muscle. Specialized cardiomyocytes were then found throughout the length of the sub-pulmonary infundibulum (Fig.8), but with none found distal to the hinges of the leaflets of the pulmonary valve. We were, however, unable to distinguish transitional cells in the micro-CT data.

Discussion

The histological identification of endocardial specialized conduction cardiomyocytes in humans has been controversial.^{21,26} Using the criteria of their exclusive endocardial localization, and their isolation within connective tissue sleeves, as established in the classical monograph of Tawara, and endorsed subsequently by Aschoff and Monckeberg, we were able to identify such cells (Figs 3A and 4A,C). The only other groups of cells scattered within the

endocardium are smooth muscle cells. Additional isolated cells with the cytological features of specialized conducting cardiomyocytes were found within the infundibulum, just below the basal attachment of the leaflets of the pulmonary valve, but with none extending into the myocardium found at the bases of the pulmonary valvar sinuses. Based on the findings from the microcomputed tomographic data, we presume that the ramifications represent the terminal of the arborizations of the right bundle branch. The ramifications themselves, furthermore, track basally into the infundibulum from the level of the base of the anterior papillary muscle. We did not find any direct ramifications from the right bundle branch at the level of the medial papillary muscle, the conducting tract retaining its insulating sheath at this level.

Proving the existence of such specialized cardiomyocytes within the infundibulum is of obvious clinical significance. It is well established that the right ventricular outflow tract is the most common source of ventricular arrhythmias in non-structural heart disease.¹⁵ Some of these arrhythmic focuses are known to be endocardial, and have been successfully ablated using radiofrequency.¹⁵ Other focuses are to be found on the epicardial aspect of the valvar sinuses.⁹ These sites are distinct from the ramifications observed in our study. To the best of our knowledge, this is the first instance in which such a rich sub-endocardial specialized network of cardiomyocytes has been demonstrated in the

subpulmonary infundibulum of the human heart.⁹ We also confirmed the presence of a comparably rich endocardial network of cardiomyocytes, with comparable histological features, at the base of the anterior papillary muscle,. The micro-computed tomographic data showed that the ramifications extended distally from this point.^{8,24} The similarity of the cardiomyocytes in both locations adds further support to our inference that the aggregates are, indeed, ramifications from the right bundle branch. We are now hoping to examine the identified aggregates in greater detail using immunocytochemistry, aiming in this way to provide still further evidence of their specialized nature.

Acknowledgments

We thank Paulo Gutierrez and Lea M M F Demarchi (Incor-SP) for useful discussions and encouragement. We also thank Ms. Sarah Gluschitz from the Department of Anatomical Sciences, St George's University, medical Illustrator for the illustrations used in this publication. The heart used prepared for microcomputed tomographic interrogation was prepared thanks to the efforts of Dr Halina Dobrynzsky and Mr Andrew Alexander of the University of Manchester. We are indebted to them for making the dataset available for our further analysis. We also wish to acknowledge the individuals who donated their bodies without whom this project would not have been possible.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Legends to Figures

Figure1. The image shows the reconstruction of the heart prepared and analysed using microcomputed tomography. The parietal walls of the right atrium and ventricle have been removed to show the septal surface. The atrioventricular

conduction axis has been reconstructed and superimposed in green, with the ramifications of the right bundle branch shown in purple. The dashed square shows the area removed in the other hearts so as to examine the junction of the moderator band and the anterior papillary muscle. Right ventricle transversal cut showing moderator band insertion at anterior free wall at base of anterior papillary muscle. Ramifications from the right bundle branch extend to the infundibulum (star), and also the supraventricular crest (asterisk). These are shown also in Figure 8.

Abbreviations: RA right atrium; Ao – aorta; RV – right ventricle.

Figure 2. The images show, in panel A, the dissection made to remove the supraventricular crest and the subpulmonary infundibulum. In panel B, we show the gross sections taken from the proximal to the distal axis of the subpulmonary infundibulum.

Figure 3 The images show the histological features of the specialized cardiomyocytes identified in the subpulmonary infundibulum. Panel A shows the scattered groups of cells separated from compact myocardium layer by collagenous endocardium. In panel B, we emphasized their “ground glass” appearance, reflecting the limited number of myofibrils and the characteristic peripheral distribution of these myofibrils around perinuclear clear area. In panel C, we show that, in general, the specialized cardiomyocytes are larger than the working cardiomyocytes in the underlying myocardium. Panel D then provides details of

two groups of specialized conducting cardiomyocytes from Panel C. Panels A and D were prepared using a double reticulum and Masson's trichrome stain, while panels B and C shows the hematoxylin and eosin stain.

Figure 4. Panel A shows the histological features of the endocardium and adjacent myocardium at the right ventricular apex in the area of fusion of the moderator band with the base of the anterior papillary muscle, as shown in the dashed area of Figure 1. Note the two groups of ensheathed cardiomyocytes with a characteristic enlarged space between the groups and the enclosing fibrous sheath. Panel B shows a false tendinous cord composed entirely of specialized conduction cardiomyocytes. Panel C is from a section taken across the supraventricular crest, while Panel D is from the subpulmonary infundibulum. Both show characteristic groups of specialized conducting cardiomyocytes. Panels A, C and D are all stained with hematoxylin and eosin and eosin, while panel B is stained using Masson's trichrome technique.

Figure 5. Schematic frontal view of the opened sub pulmonary infundibulum showing the results of the histological study done in two human hearts. In each of the rings sectioned there is graphic representation of the findings. (printed with permission from Sarah Glusnitz)

Figure 6. Panel A shows a transition between a specialized conducting cardiomyocyte and a working cardiomyocyte at the base of the anterior papillary

muscle. In panel B, we show the termination of a false tendinous cord filled with specialized cardiomyocytes to the extreme left, and its transition to working myocardium. Panels C and D shows transitional cells in the subpulmonary infundibulum.

Figure 7. The panels show the features of isolated cardiomyocytes obtained from the subpulmonary infundibulum immediately proximal to the basal attachments of the leaflets of the pulmonary valve. The isolated cardiomyocytes marked with the asterisks have morphological features fulfilling the criteria for specialized conducting cardiomyocytes.

Figure 8. The image shows a frontal reconstruction of the ramifications of the right bundle branch prepared from the heart analyzed using microcomputed tomography, and also shown in Figure 1. The myocardium has been 'ghosted' to reveal the full extent of the specialized cells. The abbreviations and coloring are as for Figure 1.

Subpulmonary Infundibulum ★

Figure1

Figure 2

Figure 3

Figure 4

Figure 5.

Figure 6.

Figure 7.

Figure 8