Morphometric analysis of the His bundle (atrioventricular fascicle) in humans and other animal species. Histological and immunohistochemical study

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ORIGINAL ARTICLE

Abstract
The His bundle is a part of the specialized electrical conduction system that provides a connection between the atrial and ventricular myocardial compartments in both normal and abnormal hearts. The aim of this study was to perform a morphometric analysis of His bundle characteristics of in humans, dogs, horses and pigs and compare them in these studied species. Histological sections of 5 μm thickness were obtained and stained with hematoxylin–eosin and Masson’s trichrome; the desmin and periodic acid–Schiff methods were also used for precise identification of cells. The His bundle was found to be longer in horses (2.85 ± 1.02 mm) and pigs (1.77 ± 0.9 mm) than in dogs (1.53 ± 0.8 mm) or humans, in which it was shortest (1.06 ± 0.6 mm). The area and diameters in His bundle cells, were significantly larger in pigs and horses than in humans (p < 0.001) or dogs (p < 0.001). We found two organizational patterns of His bundle components: group I, with large cells and a high amount of collagen fibers in ungulates (pigs and horses); and group II, with smaller cells and lower abundance of collagen fibers in humans and dogs. Documenting cell size variations in the His bundle allows us not only to identify this bundle by histological or anatomical location but also to differentiate these cells from others such as nodal or Purkinje cells. Our analysis revealed that His bundle cells have discrete identities based on their morphometric and histological characteristics.

Keywords
Conduction system · His bundle · Morphometry · Collagen fibers

Introduction
The His bundle (HB) is responsible for transmitting the electrical impulse from the atria to the ventricles where it connects with the Purkinje cells, and is the natural continuation of the AV node for rapid and synchronous activation of the ventricles. Its histological structure is difficult to identify, and special techniques are needed to identify it.

Described initially by Wilhelm His in 1893, the HB is the continuation of the atrioventricular (AV) node, from where the right and left branches of this bundle emerge, at level of the interventricular septum crest; and from where the electrical impulse is transmitted from the atria to the ventricles (Uhley and Rivkin 1960; Bishop and Cole 1967; Bharati et al. 1991; James 2002; Vigmond and Stuyvers 2016; Duan et al. 2017). The so-called HB is a part of the specialized electrical conduction system that in normal hearts provides the connection between the atrial and ventricular myocardial compartments (Tawara 1906, 2000; Cabrera et al. 2020). The findings of Tawara (1906, 2000) made it possible to pinpoint the limits of the HB (known at that time as the AV penetrating bundle). These limits begin at the transition made by the AV node when the bundle penetrates the insulating tissues of the central fibrous body and extends posteriorly to the crest of the muscular ventricular septum where it branches out (Tawara 1906, 2000; James 2002; Cabrera et al. 2020). HB cells in humans are small, pale and with few myofibrils, while in larger mammals they have the same characteristics but are larger in size (Truex and Smythe 1965; Bishop and Cole 1967; Bharati et al. 1991; Eliška 2006). HB cells in pigs and horses closely resemble Purkinje cells (PC) but are...
smaller (Mettam 1928; Glomset and Glomset 1940; Bishop and Cole 1967; Bharati et al. 1991). It has been reported that HB cells and branches have a high concentration of glycogen in dogs and horses (Uhley and Rivkin 1960; Bishop and Cole 1967).

Few studies have observed the morphometric parameters of the HB and its cells, but its structure has been described as is 1–1.5 mm in length in dogs and 0.25–0.75 mm long in humans (Ho et al. 1995). This indicates that the HB is longer in dogs than in humans, possibly because of the more extensive central fibrous body in canines (Tawara 1906, 2000; Ho et al. 1995).

HB differentiation in these different species is difficult when attempting cell identification with the naked eye by light microscopy (made according to location and characteristics of the surrounding connective tissue). Immunohistochemical staining with markers such as desmin has therefore emerged as a vital tool for accurately identifying these cells and thus facilitating precise morphometric analysis. Desmin has been shown to be the main intermediate filament (IF) present in cardiac muscle, which tends to accumulate in the intercalated and Z discs, leading to connection of the contractile apparatus with the other cellular components (Fuchs and Weber 1994; Paulin and Li 2004; Lowery et al. 2015). This IF can also be identified in larger quantity in cardiac conduction system cells than in cardiomyocytes, mainly because it can be occur throughout the cytoplasm in the former but only at specific points in the latter (Eriksson et al. 1979; Forsgren et al. 1982; Yoshimura et al. 2014).

This research underlines the importance of studying the components of the His bundle in different species and using these characteristics as a model for clinical study in human hearts. The histological characteristics of this bundle have been described, but very little objective data has been published. Accordingly, our aim was to perform morphometric analysis of HB characteristics and compare them among the four species studied.

Materials and methods

Sample processing and staining

We analyzed five hearts (medium sized, 60–80 kg) of human male adults aged 20–60 years obtained from autopsies of the Institute of Legal Medicine and Forensic Sciences of Bucaramanga, Colombia. Likewise, hearts were obtained from other animals for study: five male pig hearts (weight, 85–90 kg; average age, 5 months, cross breeds of Pietrain, Belgian Landrace, and Large White), five hearts from male horses with a height of 1.40–1.45 m, considered medium-sized animals in the American continent (height, 1.40–1.45 m; weight, 250–300 kg; age, 2.5–3.5 years; breed Colombian Creole), and five hearts of male dogs, subjected to autopsy in small animal clinics in Bucaramanga-Colombia (medium-sized adults; weight, 8–19 kg; age 5–12 years old; breeds, Schnauzer, Springer Spaniel and Creole). Procedures were in accordance with the Ethics Committee of the Universidad Cooperativa de Colombia (No. 014-2018) and comply with resolution 008430 of 1993, decree 2164 of 1992 and Law 10 of 1990 of the local Ministry of Health and the principles of the Declaration of Helsinki. Additionally, they comply with Law 84 of 1989 with a national scope, corresponding to Chapter VI of the "National Statute for the Protection of Animals", on use of animals in experiments and research.

Five atrioventricular area samples per heart were collected for histological analysis. The samples obtained for study included the junction between the interatrial and interventricular septum, the cutting area extending two centimeters on each side of the partition. Samples were fixed in a 5% formaldehyde solution, labeled for identification, and included in paraffin. Histological sections of 5 μm thickness were obtained with a microtome and stained with hematoxylin–eosin and Masson’s trichrome. For improved identification of HB cells, we also performed immunohistochemical staining with clone D33-IR606 of Anti-Human Desmin (DAKO Corporation) to visualize intermediate myofilaments (desmin) and compare them with surrounding cardiomyocytes, combined with periodic acid–Schiff (PAS) method to visualize the amount of glycogen present in these cells and facilitate identification.

Image assessment and histo-morphometric analysis

Each sample obtained was histologically and morphometrically analyzed in each stain used for the study. Samples were evaluated using a Leica DMD108 optical microscope (Leica Microsystems, Wetzlar, Germany). Computerized morphometric study was performed using Image-Pro Plus 7.0 software (Media Cybernetics, Silver Spring, MD, USA). We studied 200 micrographs (50 micrographs of the atrioventricular area for each species), where different measurements described below were made. HB length was measured from the end of compact node ends to the beginning of the bifurcation of the His branches on the crest of the interventricular septum. In the HB, we analyzed area, mean diameter, percentage of connective tissue, percentage of fundamental substance (space to be occupied by glycosaminoglycans), and percentage of cells at 4X or 10X magnification. Individually, in the HB cells and in the surrounding cardiomyocytes, we also measured area, maximum diameter, minimum diameter, mean diameter, and roundness at 20X or 40X magnification.
Statistical analysis

Descriptive statistics and hypothesis testing were performed using SPSS 20 software (SPSS, Chicago, IL, USA) and Microsoft Excel 2013. Statistical significance was set at \( p < 0.05 \). Continuous variables were expressed as mean and 95% confidence interval. Descriptive statistics were calculated for each morphometric parameter and the Kolmogorov–Smirnov normality test was performed for each sample. In case of quantitative variables when comparing two independent groups and with a small sample (fewer than 30 samples as in the HB parameters), Mann—Whitney U test was chosen, and Student T test was chosen when the sample was large (as in HB cells, where more than 50 cells per species were measured). In case of quantitative variables, after a normal distribution between species, ANOVA test was used, and when its distribution was not normal, the nonparametric Kruskal–Wallis test was chosen. Data were expressed as mean and standard deviation (SD) for all measured lengths.

Results

The mean weight of male hearts varied by species: in humans it was 300.7 ± 58 g, in pigs 360 ± 61.21 g, in horses 1.360 ± 403.8 g, and in dog hearts, 65.2 ± 13.5 g. Below we present the most relevant findings in each of the species studied.

His bundle

The HB is a thin bundle surrounded by a fine capsule of connective tissue in all the species studied and has a diameter of 0.57 ± 0.31 mm in humans, 0.44 ± 0.06 mm in dogs, 0.54 ± 0.01 mm in horses, and 0.63 ± 0.25 mm in pigs. The HB was 1.06 ± 0.6 mm in length in humans, 1.53 ± 0.8 mm in dogs, 2.85 ± 1.02 mm in horses, and 1.77 ± 0.9 mm in pigs, revealed with Masson’s Trichrome and Hematoxylin eosin technique (Table 1) (Figs. 1, 2 and 3). The percentage of collagen fibers in the HB was 36.9% of the area in humans, 25.8% in dogs, 43.2% in horses and 84.7% in pigs.
pigs (Figs. 4 and 5) (Table 1). No nerve fibers were found inside or at the periphery of the bundle in humans and dogs, whereas we found many nerve fibers inside the HB or at its periphery in horses and pigs (Fig. 3).

**Cells**

Cells were in the central portion, largely following a longitudinal and transverse arrangement, forming 2–5 cell thick
rows arranged between the collagen fibers in all species. They occupy 226.35 ± 112.17 µm² (63.1%) of the HB area in all cases in humans, 166.26 ± 63.39 µm² (74.2% of area) in dogs, 436.48 ± 330.48 µm² (56.8% of area) in horses and 530.79 ± 272.83 µm² (15.3% of area) in pigs. Cells were identified by their basophilic color within the HB and selected green in the image analyzer. CFS: cardiac fibrous skeleton; CM: cardiomyocytes; AVN: atrioventricular node.

(Table 2). The cells had a pale cytoplasm was due to its low myofibril content compared to cardiomyocytes found in the myocardium. In its cytoplasm we found abundant desmin filaments that form part of its cytoskeleton in all species. The combination of the two techniques was important in identifying these cells (Fig. 7).

This histological and morphometric analysis was performed with the aim of classifying the different components objectively. We found that HB components were organized...
into two distinct patterns: group I, with smaller cells and less abundant collagen fibers in humans and dogs; group II, with large cells and a high amount of collagen fibers in ungulates (pigs and horses).

We compared each component of the HB, finding the diameter of the His fascicle to be larger in pigs than in humans ($p = 0.035$) or in dogs ($p = 0.018$). The percentage of collagen fibers inside the HB was higher in pigs than in humans ($p = 0.001$) or dogs ($p = 0.007$), while the percentage of cells in the HB was higher in humans than in pigs ($p = 0.013$) (Figs. 4 and 5).

We next studied HB cells to uncover any differences between the species studied. Regarding HB cells, the area and diameters were significantly larger in pigs and horses than in humans ($p < 0.001$) and dogs ($p < 0.001$), and the maximum diameter of these cells was also larger in pigs than in horses ($p = 0.029$). Cells were rounder in humans than in pigs ($p = 0.049$), horses ($p = 0.036$), or dogs ($p < 0.001$).

Differentiation between HB cells in the different species showed large cells in pigs (Fig. 8a) and horses (Fig. 8b) and small cells in humans (Fig. 8c) and dogs (Fig. 8d). This provides us with alternative criteria for identification and differentiation between these cells.

### Discussion

The His bundle is the focus of increasing research interest, as the natural continuation of the conduction system from the supraventricular portion of the heart, which also results in depolarization of the large ventricular mass to the most distal portions.
Most studies reporting HB morphometry in humans show it to be a small structure, less than 1 mm in size (Tawara 1906, 2000; Ho et al. 1995; De Almeida et al. 2020). Other studies, however, have reported higher HB morphometric values in humans than those described by most authors or as in our findings. Cabrera et al (2020) indicate that the HB measures 2.68 × 3.7 mm; Kistin (1949) reports measurements of 11.3 × 1.8 mm, and Shimada and Arita (1996) indicate a HB width of 1–1.5 mm, despite that fact that the authors relied on initial descriptions by Tawara (1906) (Kawashima and Sasaki 2011). These wide variations may be due to the different sample sizes and the fact that some authors extend their measurements more distally. Tawara’s work (1906, 2000) indicated that the HB is smaller in humans than in dogs, cats, or ungulates, albeit without reporting the morphometric parameters of this bundle, only describing measurements of the right and left branches in humans, cats, and calves. Additionally, in heart of adult large-breed dogs, the HB measured 3 × 0.7 mm, which contrasts with our results found in medium-sized breed dogs.

Several studies have indicated that HB and its branches are composed mainly of PC, but smaller than those found in the final portion of the conduction system (Glomset and Glomset 1940; Bishop and Cole 1967; James 1970; James and Sherf 1971; Bharati et al. 1991; Nabipur 2004). In most studies this has been revealed via electron microscopy analysis, but differences have been found in certain characteristics, so for purposes of simplicity, here they are termed HB cells to distinguish them from the cells of the final portion of the conduction system. HB cells are larger in mammals such as horses, cows, and pigs and smaller in humans and dogs according to previous reports.
(Glomset and Glomset 1940; Bishop and Cole 1967; Bharati et al. 1991; Eliška 2006), and these cells have also been described as elongated and oblong in humans (James and Sherf 1971). These findings coincide with our study, in which cells are larger in ungulates in the HB and smaller in humans and dogs, supporting our suggested classification into two groups. In humans, the HB has been described as a cord-shaped structure with a diameter of 4 mm and a fascicle thickness of 0.7 mm (Waller et al. 1993; Randhawa et al. 2017). In our research we found the same HB structure, but with slightly lower values than previously reported.

In pigs and horses, a large number of nerve fibers have been identified inside the HB, as well as increased connective tissue compared to the surrounding cardiac fibers (Mettam 1928; Glomset and Glomset 1940; Bishop and Cole 1967; Bharati et al. 1991). In humans, nerve fibers inside the HB and at the periphery are also documented (Montoya and Ynaraja 1992; Waller et al. 1993) and little connective tissue inside the HB has been described in dogs (Montoya and Ynaraja 1992). In our study, we detected a large amount of nerve fibers present inside the HB in horses and especially in pigs but found no nerve tissue in humans or dogs. These generally sympathetic nerve fibers decrease the conduction time from the atria to the ventricles, partly owing to increased permeability of calcium ions which creates the action potential to excite the contractile process of myofibrils (Guyton and Hall 2006). We also observed a large amount of connective tissue within the HB in pigs and horses, largely generated by the central fibrous body where the bundle is located.

In dogs and horses, several authors have reported that HB cells are rich in glycogen (Uhley and Rivkin 1960; Bishop and Cole 1967). We sought to identify cells using the PAS method in the four species studied, which showed negative in all samples analyzed, suggesting that the glycogen levels in these cells are very low to zero since the test is specific for this substance. In previous studies we have found that using the same procedure, Purkinje cells do present an abundant amount of glycogen in their cytoplasm, whereas in HB cells we have not found glycogen in the cytoplasm, perhaps due to fixation defects. Using desmin as an alternative method of immunohistochemical identification, we were able to detect positive staining for HB cells compared with cardiomyocytes, allowing them to be fully identified.

Electrical impulses progress from the branches of the HB towards the Purkinje fibers to finally allow ventricular contraction, and these fibers also have a determining role in generating ventricular arrhythmias, which can be observed at the electrocardiographic level (Li et al. 2015; Aouadi et al. 2019). This underlines the importance of histological study of HB, as enhanced understanding of the cellular and tissue structure associated with cardiac physiology, can enable us to delineate the sites of arrhythmia production in different species.

Conclusions

Our main findings from this study are that HB cells are discrete entities based on their morphometric and histological characteristics. In humans and dogs, the HB has smaller cells, fewer collagen fibers, and no nerve fibers. In horses and pigs, the HB has large cells, a large number of collagen fibers, and a large number of nerve fibers. In all species, HB cells have a large number of desmin filaments.

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Author contributions FGT and ARS conceived and designed the study. FGT performed the experiments. FGT wrote the first draft of the manuscript. FGT and ARS revised the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets in this study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

The authors declare that all experiment protocols were approved by the Ethics Committee of Universidad Cooperativa de Colombia (No. 014-2018) and comply with resolution 008430 of 1993, decree 2164 of 1992 and Law 10 of 1990 of the local Ministry of Health and with the principles of the Declaration of Helsinki. Additionally, they comply with National Law 84 of 1989, which corresponds to the “National Statute for the Protection of Animals”, in Chapter VI of the use of animals in experiments and research.

Consent to participate Not applicable.

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Conflict of Interest The authors declare that they have no conflict of interests.

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