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Title: "Immunohistochemical Expression of Aldosterone Receptors in Cardiac Tissues of Healthy Dogs"

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ABSTRACT
Elevated levels of aldosterone are associated with deleterious effects on the cardiovascular system, which contributes to the development of endothelial dysfunction, fibrosis and inflammation hypertrophy, heart failure, sympathetic activation, stroke and renal dysfunction. Furthermore, it has been shown that treatment with mineralocorticoid receptor antagonists reduce the progressive damage that occurs in aldosterone target organs of patients with hypertension or heart failure, both in humans and in dogs; however, the expression of such receptors has only been demonstrated in human cardiac tissues, rabbit and rat, not so in the dog. To determine the expression of aldosterone receptors in cardiac tissues of healthy dogs, we employ the technique of immunohistochemistry for positive labeling with specific antibody in the hearts of two clinically healthy beagle dogs. Immunohistochemical assays performed, showed for first time, unpublished results of positive immunoreactivity to aldosterone receptors in dogs. This study concludes that the presence of aldosterone receptors in the heart of healthy dogs, given the existing cardio-renal axis similar to human and allows us to propose new research to test their possible alterations and pharmacological manipulation in the treatment of myocardial fibrosis reversibility using antagonists of these receptors.

Keywords: Dog Heart. Myocardial fibrosis. Immunoreactivity. Cardiomyopathy.

INTRODUCTION
For many years it is known that in the epithelial tissues, aldosterone causes retention of Na\(^+\) and water, and loss of K\(^+\) and Mg\(^{2+}\). However, aldosterone also has significant extrarenal action mediated by activation of the mineralocorticoid receptors in the heart, brain and vessels. Recently it has been demonstrated both experimentally and in clinical practice that aldosterone is an independent risk factor for developing cardiovascular diseases. Indeed, elevated levels of aldosterone are associated with deleterious effects on the cardiovascular system which contributes to the development of endothelial dysfunction, fibrosis and inflammation hypertrophy, heart failure, sympathetic activation, stroke and renal dysfunction. Furthermore, it has been shown that treatment with antagonists of mineralocorticoid receptors reduces the progressive damage that aldosterone produced in the target organs of patients with hypertension or heart failure, both in humans and in dogs[1]. The presence of excess aldosterone is an important pathophysiologic factor in left ventricular hypertrophy (LVH) and heart failure (HF), beyond the changes in blood pressure (BP) that may exist [2-4]. Found mineralocorticoid receptors (RMC) in the heart, brain and kidney of the rat, which have been cloned and have high affinity for both aldosterone and cortisol. There is a specific RMC in human cardiac myocytes [5]. Furthermore, it has been shown that the myocardium can produce aldosterone [6]. It has been reported marked decrease thanks reactive fibrosis dogs to eplerenone, aldosterone antagonist [7]. It is not clear that the aldosterone antagonists reduce cardiovascular mortality and ischemia. Aldosterone causes endothelial dysfunction in experimental animals and increases macrophage infiltration and atherosclerosis. Coronary and aortic endothelial cells (EC) express mRNA and protein RMC and RMC of the EC mediate transcription of aldosterone-dependent genes. Stimulates aldosterone ICAM-1 gene and protein expression in the EC of the coronary arteries, processes that are inhibited by spironolactone. Aldosterone promotes leukocyte adhesion to...
the EC and spironolactone inhibits this effect [8]. Another aspect to be noted is the finding of mineralocorticoid receptors in the brain that produce and sympathetic nervous system stimulation can cause increased BP and inflammatory responses [9]. Aldosterone receptor (mineralocorticoid), have been described in rabbits [10], human [11] mice [12] and rats [13], but history has not been found in dogs; perhaps, because those studies are focused on the application in human cardiology and not towards veterinary cardiology. Also, it has been reported its overexpression in human cardiovascular tissues with congestive heart failure by hybridization and immunohistochemistry [14].

The sustained levels of aldosterone in dogs, leading to various cardiac diseases such as dilated cardiomyopathy and hypertension among others, and their receptors antagonists, prevent and reduce the damage by fibrosis and inflammation; these receptors have not been mapped in the dog heart tissues, therefore study the localization and quantification of these receptors in the dog's heart, allow us to characterize the presence and distribution and then determine possible alterations in the heart of the dog CMD as well as the changes that cause expression on treatment with antagonist drugs possibly mineralocorticoid receptor regulation in its expression; knowledge that can be extrapolated to human cardiology.

MATERIAL AND METHODS

Two adult healthy Beagle dogs were used, as the experimental prototype of this kind; such animals, provided by the animal facility "Claude Bernard" of the Autonomous University of Puebla (Approval Letter No. BCB/001/2012) and treated according to the Guide for the Care and Use of Laboratory Animals of Mexico [15]. The animals underwent cardiovascular and general health assessment by auscultation, blood analysis, radiography, electrocardiography and echocardiography to ensure healthy animal status.

Lab Supplies: All reagents were from Sigma-Alcrich unless otherwise indicated (Sigma Chem Co. St. Louis Mo, USA; Aldrich Chem Co, Milwaukee, Wi, USA) H10E4C9F primary antibody (Anti-mineralocorticoid Receptor Monoclonal Antibody. Sta Cruz Labs, Ca USA), Peroxidase conjugated antibody of goat anti-mouse secondary (Jackson Labs, Las Vegas, NV, USA). Avidin-biotin complex. Diaminobenzidine substrate. Methyl green (Meyers Chemicals Inc, Tonawanda, NY, USA). Pentobarbital sodium (Vetemex, zapopan, Jal, Mexico), heparin (Piza Laboratorios, Atitalaquia, Hidalgo, México). Hartman solution (laboratorios Piza, Zapopan, Jal. México), syringes (Becton, Dickinson and Company, NJ, USA), Electrocardiographs, thermal graph paper, conductive gel, clip electrodes: Schiller AT1, Doral, Fl, USA). M7Vet Doppler echocardiograph color system (Mindray DS USA; NJ USA), Digital Radiology (C.R) (Fuji Electric Industry Co, LTD, Ky, Japan). Aneroid Sphygmomanometers (Hergom, Beijing, China) with bracelets suitable for dogs. Thermal Magnetic Stirrer (Thermo Fisher Scientific, Co. Waltham, MA, USA), Micropipettes, Micropipette tips of various capacities and Eppendorf tubes various capacities (Eppendorf Co. Hamburg, Germany). Optical Microscope slide and cover glass (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry technique in cardiac tissue by peroxidase: The animals were sacrificed using a single intravenous overdose of sodium pentobarbital (90mg/kg); the hearts were isolated by dissection and washed thoroughly in saline. Once we obtained pyramidal heart tissue sections were made and placed in 10% formaldehyde for 24 hours. After fixation of tissue were cut on vibratome sections with 50 microns thickness and were retained in sucrose. All sections were then incubated in phosphate buffer solution plus Triton X-100 (PBS Tx X - 100) 0.2% hydrogen peroxide (H2O2) at 30% for 10 minutes at room temperature to block endogenous peroxidase, were performed three subsequent washes of 10 minutes each . Were placed in blocking solution (free IgG Albumin 2%) for one hour, then three washes of 10 minutes each were performed. They were incubated for 12 hours at 4 ºC with primary antibody H10E4C9F MCR (Anti- mineralocorticoid receptor Monoclonal Antibody; Sta Cruz Labs) (1:100). The tissues with PBS solution X-100 0.2 %, all sections were incubated one hour with secondary goat antibody anti-mouse (Jackson Labs) (1:500) and were washed. After being washed were immersed in Avidin-Biotin Complex (CAB) for an hour. The labeling was developed using Diaminobenzidine substrate (50mg/100μL). Negative controls were included with PBS pH7.4 solution instead of primary antibody.
RESULTS
The animals underwent cardiovascular and general health assessment by auscultation, blood chemistry and blood count cytometry, radiology, electrocardiography and echocardiography to ensure healthy animal status. Immunohistochemical assays performed on tissue sections of heart from each of the Beagles hearts, both number 1 and number 2, shows for first time, unpublished results of positive immunoreactivity for aldosterone receptors in the four cardiac chambers of both samples (Fig. 1-5); immunoreactivity qualitatively suggests a consistent location in atria and ventricles heterogeneous, showing higher in the left ventricle compared to the right.

DISCUSSION
The first publications that gave evidence of the existence of MCR in the cardiovascular system were made by Lombes et al [10], in adrenalectomized female New Zealand rabbits, performing immunohistochemistry and competition binding studies between the antibody (H10E) and aldosterone. They found immunostaining in atria and left ventricles and aorta arteries, arterioles and capillaries. Other publications by Bonvallet et al [16], on human hearts, were used to detect the presence of intracellular MCR and the presence of the enzyme 11βHSD2 as protective mineralocorticoid. They were immuno-localized with immunohistochemical assays on left atria and ventricles. In our results, shows for first time, unpublished results of similar immunoreactivity as Lombes publications in rabbits and in humans by Bonvallet, locating the mineralocorticoid receptor with antibody H10E in the four heart chambers heart of healthy dogs.

CONCLUSIONS
In this paper, the results obtained show the existence of aldosterone receptors by immunohistochemistry in the heart of healthy dog. Obtaining a consistent location in atria and ventricles heterogeneous; being greater presence in left ventricle compared to the right ventricle. This will allow us in future studies to compare possible changes present in animals with induced and spontaneous dilated cardiomyopathy.


Conflict(s) of Interest/Disclosure(s): None.

All authors have read and approved the submission of the manuscript; the manuscript has not been published and is not being considered for publication elsewhere.

FIGURES

Figure 1. Beagle 1. A: Right atrium. The image shows immunopositivity in myocardial fibers (10x, scale bar =100µM). B: Left atrium. The image shows the immunopositivity tissue. (40x, scale bar =50µM).
Figure 2. Beagle No 1A: Showing right ventricular tissue immunopositivity (40x, scale bar =50µM). B: Left ventricular myocardial immunopositivity (40x, scale bar =50µM).

Figure 3. Beagle 2. A: Right Atrium. Immunopositivity tissue (10x, scale bar =100µM). B: Left Atrium immunopositivity tissue (40x, scale bar =50µM).

Figure 4. Beagle 2. A: Right Ventricular Myocardial immunoreactivity (40x, scale bar =50µM). B: Beagle 2. Left ventricular myocardial immunoreactivity (40x, scale bar =50µM).
REFERENCES