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研究計畫中英文摘要：

（一） 計畫中文摘要:

背　景： 心房顫動及心臟衰竭是兩種非常常見的心血管疾病，許多臨床及動物實驗皆顯示心臟衰竭會導致心房擴大、心房纖維化、及心房凋零，這些心房結構的變化導致心房顫動的產生。此外，心房的再塑作用可能是導致心房顫動引發及持續的原因，這包括細胞性、結構性及電氣性再塑。然而心臟衰竭後整個心房的電量圖改變則仍未有報導。另外，心肌細胞間隙連結(包含連結素 40 及 43 及 annexin-6)的分布、大小、及數量會影響心房的電氣傳導，以前的研究也顯示間隙連結再塑作用在心房的再塑作用及心房顫動的引發及持續扮演極重要的角色，然而心臟衰竭引起心房的間隙連結再塑作用仍是未明。因此本實驗主要在探究心臟衰竭後心房之組織病理特性。

方　法：我們在非接觸式立體定位系統偵測下，10 隻正常成狗及 10 隻心臟衰竭成狗分別將心臟取出，再接上蘭根道夫(Langendorff)模式，之後接受不同部位電刺激，記錄右心房組織電位變化，並藉由立體定位標誌電位較高及電位較低之心房組織，電生理檢查完成後將心臟自狗取出，進行組織採樣。最後使用組織顯微鏡檢、定量免疫標定與西方墨點標誌分析對不同細胞標記的分布與表現進行分析。

結　果：心電生理特性中心臟衰竭狗有較高的心房不反應期(四個心房位置皆明顯較正常狗高) ，同時心房顫動引發的機率及心房顫動引發的時間皆較正常狗來得高。在免疫共軛組織研究中，以不同切片部位連結素染色點分布總面積與總數之變異係數來評估連結素分布離散程度，其中心臟衰竭狗之連結素 43 分布，在分布總面積與分布總數上，其變異係數值均明顯高於正常狗; 而連結素 40 分布在兩組則無多大差異。

結　論：在心臟衰竭狗之心房電生理特性明顯比正常狗容易產生心房顫動，同時連結素 43 在心臟衰竭狗之右心房呈現不均勻分布，此等性質可能與心房不均勻的傳導特性與心房顫動的持續有關。

關鍵詞: 心房顫動、心房再塑、間隙連結

（二） 計畫英文摘要:

Background：Atrial fibrillation and heart failure are two very common cardiovascular disorders. Clinical and experimental studies have demonstrated that atrial dilatation with atrial fibrosis or apoptosis is considered as the structural changes of heart failure to develop atrial fibrillation. In addition, previous studies have demonstrated that atrial remodeling, including cellular, structural and electrical remodeling, may be responsible for the initiation and maintenance of atrial fibrillation. However, the voltage changes of atrial substrate in heart failure have not been defined. On the other hand, the distribution, size and amount of gas junctions (consist of connexin 40 and 43) in the cardiomyocytes may influence the electrical conduction properties of atria. Previous studies also demonstrated that gap junctional remodeling may play an important role of atrial remodeling and the maintenance of atrial fibrillation. However, the atrial gap junctional remodeling in heart failure remains unclear. Therefore, the first step of our study was to investigate the immunohistopathological characteristics of different conduting substrates.

Methods：Under the guidance of noncontact mapping system, 10 adult normal and HF dogs received electric stimulation from different sites in different coupling intervals. AF was induced from multiple pacing sites and the duration of induced AF was determined. After noncontact mapping, 8 to 10 pieces of atrial tissues were sampled from different sites of RA for analysis of gap junctions, including connexin43 (Cx43) and 40 (Cx40).

Results：In the electrophysiological properties, the 5 HF dogs had significantly higher atrial ERPs than 7 control dogs in four different atrial sites. In addition, the HF dogs had a higher AF inducibility and longer duration of sustained AF than normal dogs. In the immunoconfocal studies, the coefficient of variation (COV, standard deviation/mean X100%) of the total areas and numbers of immunolabeled Cx43 and Cx40 dots from at least 5 different views of each piece of tissue were determined. In HF dogs, the COV of total areas and numbers of Cx43 were significantly higher compared to normal dogs (HF vs. normal: Cx43- total areas 63±18% vs. 33±19%; total numbers 48±22% vs. 24±14%, both $p<0.05$; Cx40- total areas 49±22% vs. 42±16%; total numbers 47±20% vs. 39±16%, both $p>0.05$).

Conclusions: HF dogs had more electrophysiologic properties to maintain AF, and the RA gap junction expression was also more heterogeneous, which might relate to the heterogeneous conduction properties and maintenance of AF.

Keywords: atrial fibrillation, atrial remodeling, gap junction

Introduction:

Atrial fibrillation (AF) and heart failure (HF) are two very common cardiovascular disorders, and both are associated with significant morbidity and mortality (1-2). AF may worsen the morbidity and mortality of HF and HF may predispose the development of AF. Clinical and experimental studies have demonstrated atrial dilatation in HF, and atrial dilatation may result in atrial fibrosis or apoptosis, which is considered as the structural changes of HF to develop AF (3-6) In addition, previous studies have demonstrated that atrial remodeling, including cellular, structural and electrical remodeling, may be responsible for the initiation and maintenance of AF (3-7). However, the voltage changes of atrial substrate in HF that predispose to AF have not been determined.

Non-contact three-dimensional mapping system (EnSite) has been used to improve the mapping and ablation of tachyarrhythmias, especially in complex atrial tachyarrhythmias after surgically corrected congenital heart diseases (8-10). Identification of target sites for ablation is challenging, because reentrant circuits of atrial tachyarrhythmias are complex. The critical sites in these patients are often slow conduction zone (protected isthmus) bordered by scar tissue. EnSite can provide isopotential map with color settings to display the detailed three-dimensional activation mapping for investigating the arrhythmogenic foci or reentrant circuits. In addition, it can also provide global voltage map to define the low voltage zone. Voltage mapping has been developed to allow discrimination of these slow conduction zones from surrounding scar tissues (11). The introduction of three-dimensional mapping systems facilitated the voltage mapping and reconstruction of complex reentrant circuits.

On the other hand, in mammalian heart, there exist gap junctions in specialized plasma membrane regions of atrial cardiomyocytes, and gap junctions in the cardiomyocytes seem to play an important role by forming a direct communicating channel between adjacent cells. Distribution, size, and amount of gap junctions may influence the electrical conduction properties (12-13). Gap junctions in mammalian atrial cardiomyocytes mainly composed of 2 types of connexin (Cx) proteins, Cx 40 and Cx43. In 1994, Saffitz et al showed that the different distribution and characteristics of gap junction Cx protein may determine the different conduction properties presented in atria and ventricles (14). In 2000, van der Velden et al showed that in rapid pacing model, the distribution patterns and the amounts of Cx 40 changed progressively, and the increased Cx 40 distribution heterogeneity correlated with the increased AF stability and structural atrial remodeling (15). Therefore, gap junctional remodeling may play an important role in atrial remodeling and the maintenance of AF. However, the atrial gap junctional remodeling in heart failure remains unclear.

Therefore, the purposes of the present study were to investigate the changes of global voltage mapping and the gap junction distribution of both atria in dogs with HF.

Methods

Animal preparation

The protocol for animal preparation was approved by the Committee for Experiments on Animals of the Taipei Medical University. Forty mongrel male dogs (weight, 20-30 Kg), 20 control and 20 study dogs, are included. No intervention is performed before experimental protocols in the

normal dogs. Study dogs undergo pacemaker implantation to result in pacing-induced heart failure, and then experimental protocols were performed. The dogs were anesthetized with ketamine (10-20 mg/kg) and sodium pentobarbital (30 mg/Kg intravenous). They were artificially ventilated through a cuffed endotracheal tube by a constant volume cycled respirator with room air. A right lateral thoracotomy was performed, and a right ventricular (RV) free wall pacing lead was sutured. A subcutaneous pocket was fashioned for the placement of a VVI pacemaker (Activitrax or Spectrax, Medtronics). After complete the study, dogs were allowed to recover fully with proper care for 7 days. Then, a rapid pacing model was initiated at 230 bpm for 2 to 4 weeks. Pacing-induced heart failure is defined by symptoms of lethargy, loss of appetite, dyspnea and/or ascites. After heart failure developed, the dogs underwent the following experimental protocols.

Experimental protocols

1. Electrophysiologic study:

Four bipolar hook electrodes were attached to the RA appendage (RAA), middle and lower RA (MRA and LRA), and left atrial appendage (LAA) to stimulate atrial tissue. Programmed electrical stimulation is performed by a custom-made stimulator (Model 5325, Metronic, Ltd., Minneapolis, USA) that delivered constant-current pulses of 1-ms duration. The AERP is determined during atrial pacing (stimulus strength, 2 X threshold) at a cycle length of 300 ms and extrastimulation (S1S2) pacing. S1S2 pacing starts from 300/190 msec and decreases by 10 msec intervals. The longest S1S2 interval that results in a nonpropagated atrial response is taken as the AERP. After S1S2 pacing, high current (10 mA) burst pacing from all pacing sites is performed to induce AF. The occurrences and duration of AF paroxysms are recorded.

2. Noncontact voltage mapping:

After the electrophysiological study, the heart was rapidly removed and placed in cold perfusion fluid. The aorta was cannulated, and the heart was perfused at a pressure of 65 mmHg and a temperature of 37℃. The compositions (in mmol/L) of the perfusion fluid are NaCl 130, NaHCO3 24.2, KCl 4.0, CaCl2 2.2, MgCl2 0.6, Na2HPO4 1.2, and glucose 12. The perfusion fluid was gassed with a mixture of 95% oxygen and 5% CO2, resulting in a pH of 7.4. To maintain the atrial pressure for noncontact mapping, the caval and pulmonary veins were ligated and the perfusion fluid entering the right atrium from the coronary sinus was allowed to leave the heart exclusively through a cannula in the pulmonary artery. The SVC was cannulated with a Y-shaped tube for insertion of noncontact mapping balloon. To balance the pressures of both atria, the interatrial septum was perforated. We had no indication that perforation of the atrial septum affected the vulnerability of the atria for AF. To avoid variation in atrial pressure by contraction of the ventricles, 10 mmol/L butanedione monoxime (DAM) was given to reduce myocardial contractility.

The noncontact mapping system (EnSite 3000, Endocardial Solutions, Inc., St. Paul, MN, USA) consists of an inflatable multielectrode array (MEA) catheter, a reference electrode, amplifiers, and a Silicon Graphics workstation. Raw data detected by the MEA was fed to the Silicon Graphics workstation via an amplifier. The MEA was positioned in the RA chamber and inflated for mapping. The system locates any catheter in relation to the MEA using a “locator” signal, which was used to construct a three-dimensional computer model of the virtual endocardium, providing a geometry matrix for the inverse solution, and to display and track the

position of the catheter on the virtual endocardium. Using mathematical techniques to process the potentials detected by MEA, the system was able to reconstruct more than 3000 unipolar electrograms simultaneously and superimpose them onto the virtual endocardium, producing isopotential maps with a color range representing voltage amplitude. On the isopotential maps, a wave front was defined as a discrete front of endocardial depolarization presenting as a region of negative polarity.

Atrial voltage analysis

In all dogs, peak-negative voltage (PNV) was analyzed from the negative unipolar electrograms, obtained from 256 simultaneous virtual sites, equally distributed throughout the RA. Analysis is performed during sinus rhythm, atrial pacing (from four sites, RAA, HRA, LRA, LAA, with cycle lengths of 350 and 250 ms), and during AF. The mean PNV of the global RA was compared among normal and study dogs during various rhythms to investigate substrate characteristics using an offline analysis program. Global voltage distribution of global RA representing various relative ratios to the maximal PNV of during an atrial cycle was shown on the normalized flat voltage map. Areas with reconstructed unipolar voltage less negative than 20, 25, 30, and 35% of the maximal peak negative voltage were delineated and compared. Contact electrograms verified the activation path with small, fractionated unipolar potentials recorded inside the LVZ, split potentials along the LVZ border, and large, sharp potentials outside the LVZ. The mean voltage of unipolar virtual electrogram inside the LVZ and outside the LVZ, and the mean voltage reduction were also calculated during different rhythms. The program also determined the distribution of distance from the virtual sites to the balloon center and the total endocardial surface of the both atria for validation of the accuracy of reconstructed electrograms.

Activation mapping

We began analysis with default high-pass filter setting of 2 Hz to preserve components of slow conduction on the isopotential map. The activation mapping was applied by stepping the isopotential map display forward through time from the onset of activation and simultaneously drawing the path of the activation wavefront. Color settings were adjusted so that the color range matched one-to-one with the milivolt range of the electrogram deflection of interest.

3. Tissue sampling and processing:

Under the guidance of noncontact mapping, 8 to 10 pieces of atrial tissues are sampled from anterior, lateral, medial and posteroseptal walls of RA. All specimens are stored in liquid nitrogen before cryosectioning. Selected specimens are fixed in formalin and prepared for histological examination.

Immunohistochemistry:

Anti-connexin antibodies and other cell markers

For single labeling, affinity-purified rabbit polyclonal antisera against Cx40 [designated as Y21Y] and Cx43 [R-530], are used for the immunofluorescence detection of the gap-junctional proteins. For double labeling, Cx43 are labeled with mouse monoclonal antisera (BD transduction). Cardiomyocytes are identified with mouse monoclonal antiserum against α-actinin and vinculin (Sigma).

Secondary antibody/detection systems

Donkey anti-rabbit and anti-mouse immunoglobulins conjugated to either CY3, CY5 are used. CY3-conjugated antibodies are used for single labeling and 1 CY3-conjugated plus 1

CY5-conjugated for double labeling. In experiments in which 1 connexin is visualized with the anti-rabit CY3, simultaneous cardiomyocyte marking is detected with the anti-rabit CY5.

Immunolabeling of connexins

For single labeling of one connexin type, cryosections of the samples are blocked in 0.5% BSA (15 minutes) and incubated with rabbit anti-Cx40 (1:50), anti-Cx43 (1:200) at 37℃ for 2 hours. The samples are then treated with CY3-conjugated secondary antibody (1:500, room temperature, 1 hour). When simultaneous marking of cardiomyocytes is carried out, single connexin-labeled samples are incubated with anti-α-actinin (1:300) followed by treatment with the 2 secondary antibodies. Finally, the sections are mounted. All experiments included RA sections as positive controls and omission of primary antibody as negative controls.

Confocal laser scanning microscopy

Immunostained samples are examined by confocal laser scanning microscopy with a Leica TCS SP. The images from sections of multiple labeling are taken with either simultaneous or sequential multiple channel scanning. For determination of the size of and the special relationship between individual cardiomyocytes, in samples labeled with anti-vinculin, consecutive optical sections taken at 0.5-μm intervals through the full thickness of cardiac muscle, in which the long axis of the cells lies horizontal to the sections, are used. Quantification of gap junction aggregations, defined as the total areas and numbers of aggregations of Cx-labeled spots in linear or disk shape between the cells, is conducted in samples double-labeled for Cx and vinculin by use of similar principles. Quantification is performed with QWIN image analysis software (Leica). In each specimen, at least 5 images from randomly selected areas are scanned for analysis. The COV of the total areas and numbers of Cx-labeled spots in each specimen are calculated after quantification of gap junction.

Results

Animal survivals

Complete studies were performed in 7 normal dogs and in only 5 heart failure dogs.

Electrophysiological Study

The AERP in four different atrial sites were significantly longer in HF dogs than control dogs. (144±24 vs 116±16 ms in HRA, 138±20 vs 110±12 ms in MRA, 148±28 vs 120±15 ms in LRA and 134±20 vs 118±14 ms in LAA, all $P<0.05$). The degree of AERP dispersion was also significantly larger in HF dogs than control dogs (28±14 vs 12±8 ms, $P<0.05$). The inducibility of AF was evaluated by calculating total AF paroxysms divided by total induction attempts. The inducibility of AF (longer than 8 beats) (88% vs 54%, $P<0.05$) and sustained AF (longer than 10 seconds) (42% vs 18%, $P<0.05$) was more easy in HF dogs than normal dogs. The total durations of AF paroxysms were also significantly longer in HF dogs than in normal dogs (148±66 vs 24±14 seconds, $P<0.01$).

Light Microscopy

The average thickness of the red-stained myocardium was mildly thicker in anterior walls, as well as lateral, medial, and posteroseptal walls of RA in both HF than control dogs.

Immunohistochemistry and Confocal Laser Scanning Microscopy

The Cx-labeled spots in adult mongrel dogs were mostly linear, circular or discoid in shape representing gap junction aggregates in intercalated discs at the end of cardiomyocytes. In HF

dogs, the Cx43 expression was found to be heterogeneous in the cardiomyocytes. In order to evaluate the degree of the heterogeneity of gap junction expression, the total areas and total numbers of Cx-labeled spots in each scanned image were quantified. The COV of the total areas and numbers in different views of the same specimen is calculated as mean/SD X 100%. For Cx43, the COV of total Cx-labeled areas was significantly higher in HF dogs than in control dogs. For Cx40, the COV of total Cx-labeled areas was similar in both groups. In addition, for Cx43, the COV of total Cx-labeled spot numbers was also significantly higher in HF dogs than in control dogs, and for Cx40, the COV of total Cx-labeled spot numbers was also similar in both groups.

Summary

AF in HF canine model

In our study, the electrophysiological properties in HF dogs were significantly different to control dogs. The inducibility of AF paroxysms was significantly higher in HF dogs, and the duration of sustained AF was also significantly longer in HF dogs than the control dogs.

Heterogeneity in Gap Junction Expression

In the immunoconfocal study, Cx43 expression was showed to be more heterogeneous in HF dogs than in control dogs, while Cx40 expression remained similar in both groups.

Conclusion

In dogs with tachycardia induced cardiomyopathy, the RA gap junction expression was heterogeneous, which might relate to the heterogeneous conduction properties and contribute to the maintenance of AF.

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計畫成果自評:

1. 本計畫順利建立心臟衰竭動物模式，並利用三度空間非接觸影像系統於藍根道夫模式下進行動物實驗。
2. 本年度已成功分析右心房之間隙連結在心臟衰竭狗的右心房有不均勻分布。
3. 下一步將同時探討左右心房之心電生理特性與病理特徵之差異。