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行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告
□期中進度報告

犬心臟衰竭之心房的電生理及病理變化:探討心房顫動的機制（第一年）

Electrophysiology and pathology of atria in a canine heart failure model: implications for mechanisms of atrial fibrillation. (First year)

計畫類別：■ 個別型計畫　□ 整合型計畫
計畫編號：NSC 93－2314－B －038－034
執行期間：　93 年　8 月　1 日至　94 年　7 月　31 日

計畫主持人：謝敏雄
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計畫參與人員： 劉篤穎

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

執行單位：臺北醫學大學醫學系

中　華　民　國　94 年　10 月　31 日

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)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。因此本實驗第一階段主要在探究不同傳導介質之組織病理特性。

方　法：我們在非接觸式立體定位系統偵測下，10 隻正常成狗分別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：病理組織分析利用 Trichrome stain，電位高之檢體較電位低之檢體具有較厚的心房組織；PAS 鏡檢中，不同檢體其酐醣含量並無明顯差別；RS red stain 中，不同檢體其膠原纖維含量並無明顯差別。免疫標定分析：十組（電位高與電位低）有效冷凍組織切片分析中，電位高之組織其 Annexin-6 含量較高者佔七組(70%)，Connexin-43 含量較高者佔五組(50%)，Connexin-40 含量較高者佔三組（30%）。西方墨點標誌分析：在包涵 Annexin-6, Connexin-43,40, Na/Ca channel,及 Na channel 等標記之分析中，初步結果顯示高低電位組織並無明顯差異。

結　論：在成狗右心房組織電氣活動中存在不同去極化電位特性的組織，形成具有異質性之電氣傳導介質，而其組織厚度變異性可能成為決定組織傳導特性的重要因素。

關鍵詞: 心房顫動、心房再塑、電氣傳導、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 dogs received electric stimulation from different sites in different coupling intervals. The activation sequences were recorded for voltage analyses. With the navigation function of the noncontact mapping system, we marked the atrial tissues with constant high and low voltages, and sampled those marked atrial tissues after electrophysiological study. Light microscopic studies with different stains techniques, confocal laser scanning microscopy using quantitative immunolabeling, and Western blotting analyses were performed for the sampled tissues to analyze the tissue characteristics and the distribution and presentation of different cell markers.

Results：The histopathological analyses of Trichrome stain showed that tissues with high voltages were thicker than tissues with low voltages. In PAS study, no significant differences in glycogen stores were found within tissues of different voltages. In RS red stain study, no significant differences in amounts of collagen fiber existed in different tissues. The immunolabeling and confocal laser scanning microscopy showed that in 10 effective sets of frozen section specimens (high voltage vs. low voltage), 7 sets (70%) showed higher Annexin-6 levels in high voltage tissues, 5 sets (50%) showed higher Connexin-43 levels in high voltage tissues, and 3 sets (30%) showed higher Connexin-40 levels in high voltage tissues. The Western

blotting analyses using markers labeling Annexin-6, Connexin-43 & 40, Na/Ca channel, and Na channel for analyses revealed no significant differences between different specimens.

Conclusions: There were different tissues within the right atria of adult dogs, which demonstrated different depolarizing potentials during electric activation and formed heterogenous substrates for atrial conduction. The heterogeneity in atrial thickness could be important factor that determines the conducting properties of the atrial tissues.

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.

In the first step of our study, we tried to correlate the conduction properties of the atrial substrates with their immunohistopathological characteristics. Then we planned to define the electrical and pathological changes occurring during the remodeling process in the heart failure models of atrial fibrillation.

Methods

Electrophysiological study and noncontact mapping:

Ten mongrel dogs weighing 20 to 25 kg were premedicated with intravenous ketamine (10-20 mg/kg) and anesthetized by intravenous sodium pentobarbital (30 mg/kg). Then they received intubation and mechanical ventilation. Using a midline sternal incision, the chest was opened, and

the heart wass exposed for introduction of noncontact mapping and navigating catheters, suture of electric pacing wire, and marking areas of interest.

Noncontact catheter systems:

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. The MEA is positioned in the right atrial (RA) chamber and inflated for mapping. 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.

Voltage mapping

Complete RA voltage maps are obtained during SR, atrial pacing, and AF to ensure detailed voltage description of substrate. The areas of LVZ were defined as areas of abnormal voltage with a unipolar negative voltage lower than 30% of the maximal peak negative voltage (PNV). The border of the low voltage zone (LVZ) was determined along the path where activation preferentially diverted around an apparent obstacle containing voltage lower than 30% of PNV.

Electrophysiologic study:

Five bipolar hook electrodes were attached to the upper, middle, and lower parts of RA anterior wall, RA appendage (RAA), left atrial appendage (LAA) to stimulate atrial tissue. Programmed electrical stimulation was performed by a custom-made stimulator (Model 5325, Metronic, Ltd., Minneapolis, USA) that delivered constant-current pulses of 1-ms duration. Atrial pacing (stimulus strength, 2 X threshold) at cycle lengths of 350, 250, and 200 ms from five pacing sites was performed for voltage mapping. Burst pacing and/or high current pacing were also used to induce atrial fibrillation. Total episodes and duration of atrial fibrillation were recorded.

Tissue sampling and processing:

On-line analyses of noncontact voltage mapping for each activation sequence during sinus rhythm and atrial pacing from different pacing sites, atrial tissues were marked as areas with fixed low voltage zones and fixed high voltage zones. After electrophysiological study, the heart was removed and washed with heparin containing cold saline. Tissue sampling was performed in each marked zone. Tissues were divided into three parts, one part was fixed in 10% formalin for light microscopy, the second and third parts were embedded and stored in liquid nitrogen before cryosectioning.

Immunohistochemistry:

Anti-connexin antibodies and other cell markers

Affinity-purified rabbit polyclonal antisera against Cx40 [designated S15C(R83)] and Cx43 (C16), were used for the immunofluorescence detection of the gap-junctional proteins. Cardiomyocytes were identified with mouse monoclonal antiserum against α-actinin and vinculin (Sigma). Affinity-purified mouse monoclonal antisera against Na/Ca channel and rabbit polyclonal antisera against annexin-6 were used as other cell markers for different specimens.

Secondary antibody/detection systems

Donkey anti-rabbit and anti-mouse immunoglobulins conjugated to either CY3, CY5 were used. CY3-conjugated antibodies were used for single labeling and 1 CY3-conjugated plus 1 CY5-conjugated for double labeling. In experiments in which 1 connexin was visualized with the anti-rabit CY3, simultaneous cardiomyocyte marking was detected with the anti-rabit CY5.

Immunolabeling of connexins

For single labeling of one cell marker, cryosections of the samples were blocked in 0.5% BSA (15 minutes) and incubated with rabbit anti-Cx40, anti-Cx43, anti-annexin6, and mouse anti-Na/Ca channel antibody at 37℃ for 2 hours. The samples were then treated with CY3-conjugated secondary antibody (1:500, room temperature, 1 hour). When simultaneous marking of cardiomyocytes was carried out, single connexin-labeled samples were incubated with anti-α-actinin followed by treatment with the 2 secondary antibodies. Finally, the sections were mounted.

Confocal laser scanning microscopy

Immunostained samples were examined by confocal laser scanning microscopy with a Leica TCS SP. The images from sections of multiple labeling were 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, were used. Quantification of gap junction aggregations, defined as the number of aggregations of Cx43-labeled spots in linear or disk shape between the cells, was conducted in samples double-labeled for Cx43 and vinculin by use of similar principles. Quantification was performed with QWIN image analysis software (Leica).

Results

Total 10 dogs were collected tissues fixed in formalin. In Trichrome stain analyses, the thickness of sampled atrial issues was around 0.56 to 3.00 mm. The thickness ratios of low voltage vs. high voltage areas were 0.36 to 0.88 (0.62±0.20). In PAS study, dark stained glycogen-rich tissues were randomly embedded in the cardiomyocytes of atrial walls and showed no significant differences between low and high voltage tissues. In RS red stain study, no significant differences in amounts of collagen fiber existed in different tissues. Toal 10 frozen section specimens (high voltage vs. low voltage) were collected for immunolabelling and semiquantitative confocal laser scanning microscopy. There were 7 sets of atrial tissues showing higher annexin-6 staining signals in high voltage tissues than in low voltage tissues. Semi-quantitative measurement of the densities of the connexin 43 and 40 were performed and revealed only 5 of 10 sets of tissues showing more connexin-43 signals and 3 of 10 sets of tissues showing more connexin-40 signals in high voltage tissues than in low voltage tissues. Finally, the anti-Na/Ca channel antibody stained over the whole surface of cardiomyocytes in atrial tissues, as the anti-annexin-6 did. No significant differences were found between high and low voltage tissues. The Western blotting analyses were also performed. However, the preliminary data revealed no significant differences between different specimens.

Conclusions:

There were different tissues within the right atria of adult dogs, which demonstrated different depolarizing potentials during electric activation and formed heterogenous substrates for atrial conduction. The heterogeneity in atrial thickness could be important factor that determines the conducting properties of the atrial tissues.

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計畫成果自評:

1. **本計畫建立動物模式在多方嘗試失敗下才達成, 但只進行初步的實驗—正常成狗的實驗。**
2. **本計畫主要目的是想藉由三度立體空間影像定位系統所定出的低電位區與組織切片的病理特徵之間找出相關聯結,目前已有初步成果。**
3. **下一步將探討心房之心電生理特性與低電位區及組織切片的病理特徵之相互關聯,以及心臟衰竭模式及正常成狗之間的差異。**