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

（計畫名稱）

經由動物僧帽瓣閉鎖不全探討心肌細胞再未分化的機轉及時序(Temporal evolution of dedifferentiation of atrial cardiomyocytes in a pig model of mitral regurgitation: changes in atrial histopathology and signalling mediators)

計畫類別：■個別型計畫　□整合型計畫

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請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標: 學術期刊發表 (Jen-Ping Chang, Tzu-Hsien Tsai, Yung-Lung Chen, Ya-Hui Wang, Wan-Chun Ho, Guo-Hua Yu, Mien-Cheng Chen. Left Atrial enlargement induced by pure mitral regurgitation: Time frame in a new swine model. Eur Surg Res 2010;45:98-104 **(correspondence)**)

□ 未達成目標（請說明，以 100 字為限）

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2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

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3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以500字為限）

1. 學術期刊發表
2. Mitral regurgitation is one of the major underlying causes of heart failure. This swine model of pure mitral regurgitation can be used to investigate the molecular mechanisms of myolysis, which may contribute to atrial contractile dysfunction and atrial dilatation in this disease entity.

研究成果

1.Original aims/goals and accomplishments

The original aims of this study included

1. We planned to establish an animal model of mitral regurgitation and examine the time course of mitral regurgitation-induced cellular structural remodeling in atria.
2. We planned to examine the role of renin-angiotensin-aldosterone system in the genesis of dedifferentation of atrial cardiomyocytes in mitral regurgitation.
3. We planned to investigate whether the AT1 or AT2 receptor is involved in the genesis of dedifferentation of atrial cardiomyocytes in mitral regurgitation.

(1) Accomplishments:

Left Atrial Enlargement Induced by Pure Mitral Regurgitation: Time Frame in a New Swine Model

Mien-Cheng Chen, MD, Jen-Ping Chang, MD

Background: Atrial enlargement occurs in patients with significant mitral regurgitation. However, the time frame of the development of atrial enlargement induced by mitral regurgitation remains unknown.

Methods: Fourteen Lanyu miniature pigs (age, 6.6 ± 0.9 months) were studied. Mitral regurgitation was created by placing a predefined hole on the middle scallop of the posterior mitral leaflet under cardiopulmonary bypass. The parasternal long-axis atrial dimension was measured by transthoracic echocardiographic examinations.

Results: All animals exhibited grade 3 mitral regurgitation immediately after surgery. Seven pigs expired within 2 weeks after the operation [technical complications (n = 1), acute cardiac tamponade (n = 1), and acute and subacute heart failure (n = 5)]. Seven pigs remained alive at a mean follow-up of 7.7 ± 2.1 months. The left atrial diameter indices of the seven pigs increased significantly at 1 month (33.1 ± 8.6 mm, p = 0.018) and 3 months (41.3 ± 12.6 mm, p = 0.018) after surgery compared with baseline values (22.8 ± 5.2 mm), and the left atrial diameter index increased significantly at 3 months compared to 1 month (p = 0.018).

Conclusions: Left atrial enlargement develops rapidly and progresses after the creation of significant pure mitral regurgitation.

Introduction

Mitral regurgitation is one of the major underlying causes of heart failure. Atrial myocardial stretch caused by volume overload due to mitral regurgitation may trigger the development of atrial contractile dysfunction and atrial enlargement. Conceivably, persistent volume load may cause myocytes to undergo structural remodeling. Indeed, our previous studies showed that myolysis, hypertrophy, dedifferentiation, programmed cell death pathway activation of atrial cardiomyocytes, and increased oxidative stress of atrial myocardium occur in patients with mitral regurgitation. This structural remodeling should play an important role in atrial myocardial dysfunction. However, the time frame of the development of mitral regurgitation-induced structural remodeling in the atria and the molecular mechanisms (signaling mediators) of the structural

remodeling remain unknown. In an effort to understand the role of volume load caused by pure mitral regurgitation on atrial structural remodeling, we developed a new swine model of pure mitral regurgitation. Additionally, this study examined the short-term effects of pure mitral regurgitation on left atrial and ventricular sizes.

Materials and Methods

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council and published by the National Academy Press (1996). This study protocol was approved by the Laboratory Research Animal Review Committee of our institution.

Surgical Procedure for Mitral Regurgitation

Fourteen Lanyu miniature pigs (5 male, 9 female; age, 6.6 ± 0.9 months) with a mean body height of 110.3 ± 5.8 cm (range, 102 to 117 cm) and a mean body weight of 25.6 ± 3.9 kg (range, 21.5 to 33.0 kg) were included in this study. The surgical procedures were performed with standard surgical equipments as in human cardiac surgery. The surgical equipments included cardiopulmonary bypass machine (Sarns Modular Perfusion System 8000, Terumo Cardiovascular Systems Corporation Ann Arbor, MI, U.S.A.), heater cooler (Terumo TCM II, Terumo Cardiovascular Systems Corporation, Ann Arbor, MI, U.S.A.), and oxygenator (Medtronic Minimax Plus, Medtronic Inc. Mineapolis, MN, U.S.A.). Pigs were premedicated with thioamylal sodium (5 mg/kg, intramuscularly). General anaesthesia was induced with cisatracurium (0.05 mg/kg), followed by 14 French cuffed endotracheal tube intubation with ventilation management of 15 mL/kg and FiO2 of 100%, and maintained with sevoflurane inhalation (2 -4%) plus

fentanyl citrate and cisatracurium. The femoral artery was cannulated for hemodynamic monitoring and retro-auricular and femoral veins were cannulated for venous access.

A median sternotomy was performed with meticulous mediastinal dissection to maintain the bilateral pleura intact. A pericardial cradle was created, and the right atrial, pulmonary, and left atrial pressures were measured. Cardiopulmonary bypass was performed by standard moderate hypothermic (26℃) and crystalloid cardioplegic solution (Plegisol, Hospira Inc., Lake Forest, IL, U.S.A.), and cardiac arrest was instituted with ascending arch (12 French Aortic Cannula, Baxter Research Medical Inc., Midvale UT, U.S.A.) and right atrial (24 French Thin-Flex Single Stage Venous Cannula, Edward Lifesciences LLC., Irvine, CA, U.S.A.) cannulation with a perfusion flow of 70-80 mL/kg/min. In Lanyu miniature pigs, the left atrium is located at the left postero-inferior site of the heart. Hence, a transatrial approach to the mitral valve was impossible through the median sternotomy. Therefore, the mitral apparatus was approached via an apical left ventriculotomy and a 2.8 mm hole was created with a coronary punch on the middle scallop of the posterior mitral leaflet to achieve a left atrial pressure of 10-18 mmHg and create pure moderate to severe mitral regurgitation, as determined by the epicardial echocardiography (Figure 1). Color flow Doppler was used to qualitatively grade (0-4) the mitral regurgitation on the basis of color Doppler jet size and extent. By placing a predefined hole on the middle scallop of the posterior mitral leaflet, we were able to create a persistent mitral regurgitation model. All animals showed grade 3 mitral regurgitation immediately after the operation. Then, the left ventriculotomy was repaired with a Teflon felt buttress running suture. After restoring heart function, the right atrial, pulmonary, and left atrial pressures were measured. Two silastic Penrose drains were placed in the posterior pericardial and anterior mediastinal spaces to avoid the development of cardiac tamponade (except for the first two cases). The sternotomy

was closed and the animal was then stabilized and recovered from anesthesia. All animals were extubated at the operating theater.

Three 9-month-old Lanyu miniature pigs without having surgery served as normal controls.

Postoperative Management

Intensive postoperative management was implemented, including Dopamine (2-5 microgram/kg/min) and Furosemide (0.4 mg/kg q12h) for 2 weeks or until all signs of heart failure (tachypnea, orthopnea, anorexia, and abdominal distension) disappeared. The postoperative pain management was done by continuous intravenous administration of Ketorolac Tromethamine at a rate of 0.2 m g/kg/hour. The pigs were fed with 200 gm regular meal, containing cornmeal, soy beans and animal fat, twice a day after surgery.

Follow-Up Protocol

All animals were followed for clinical signs of heart failure (tachypnea, lethargy, and anorexia). One month and 3 months after surgery, the pigs were premedicated with thioamylal sodium (2-5 mg/kg, intramuscularly) before performing follow-up echocardiographic studies to evaluate the short-term effects of the newly developed pure mitral regurgitation on the left atrial and ventricular sizes. Preoperative and postoperative cardiac parameters including chamber dimensions, valvular lesions, and left ventricular function were obtained by transthoracic echocardiographic examinations using a 2.5 MHz transducer attached to a commercially available echo Doppler machine (Sonos 7500; Hewlett-Packard; Palo Alto, CA, USA).

Specimen Storage

Three pigs had small pieces of atrial tissues of the left atrial appendage (apical portion) obtained during surgery and at 3 months after surgery. Atrial tissues were fixed immediately following excision and maintained in 4% buffered formalin overnight at room temperature, then embedded in paraffin and stored until later study for hematoxylin, eosin and Masson's trichrome.

Histological Analysis

Tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol. Slides were then stained with hematoxylin, eosin, and Masson's trichrome. To quantify the quantity and percentage of myocytes with myolysis, two sections per atrial sample were analyzed, with the analysis including at least 100 randomly selected cells per section. Areas containing the maximum number of myocardial cells were selected for analysis. Tissue sections were observed under an Olympus BX51 microscope and four random images were taken under 400 X magnification. All images of each specimen were captured using an Olympus DP70 camera. Atrial cardiomyocytes were analyzed (UTHSCSA, Image tool, Version 3.0) and scored by morphometry as mildly myolytic if <10% of the sarcomere content was absent and as moderately-to-severely myolytic if >10% of the sarcomere was absent. Moreover, fibrotic areas and sizes of atrial cardiomyocyte in tissue sections were analyzed.

Measurement of Plasma B-type Natriuretic Peptide Concentrations

Peripheral venous plasma levels of B-type natriuretic peptide (Triage BNP, Biosite, CA, U.S.A.) were measured.

Statistical Analysis

Data are presented as means ± standard deviations (SD). Chamber size indexed to the body surface area was used to adjust for body size. Continuous variables were compared by Mann-Whitney U test, or

Wilcoxon signed rank test for paired data. Continuous variables at different time points were analyzed by Kruskal-Wallis test followed by Wilcoxon signed rank test. All p values were two-sided, and the level of statistical significance was set at 0.05.

Results

Table 1 lists the hemodynamic data of the study pigs. The mean left atrial pressure increased immediately after creation of pure mitral regurgitation compared with the baseline value (12.7 ± 6.3 vs. 9.7 ± 3.7 mmHg), although the difference did not reach statistical significance ($p = 0.058$). The mean right atrial pressure did not significantly change immediately after creation of pure mitral regurgitation compared with the baseline value (5.4 ± 2.9 vs. 5.2 ± 2.8 mmHg). The mean pulmonary artery pressure increased immediately after creation of pure mitral regurgitation compared with the baseline value (18.8 ± 6.4 vs. 16.9 ± 5.0 mmHg), although the difference did not reach statistical significance. The first pig expired due to technical complications. In this case, we tried to approach the mitral valve through transseptal, lateral left atrial, and left atrial appendage approaches, but in vain. This pig finally died of profound heart failure due to prolonged ischemic time. According to this experience, we brought to a conclusion that the left ventriculotomy was the only optimal route for the exploration of the mitral valve in Lanyu miniature pig model and this approach also allowed the left atrial tissue to remain intact for histological and biochemical studies. The second pig died from acute cardiac tamponade. One pig died from acute heart failure (acute pulmonary edema) 1 week after surgery. Four pigs died due to subacute heart failure (poor appetite, fatigue, and dyspnea) 2 weeks after surgery. Thus, seven pigs (4 male, 3 female; age, 6.1 ± 0.4 months) remained alive at a mean follow-up of 7.7 ± 2.1 months.

One pig had regression of mitral regurgitation to grade 2 as determined 1 month and 3 months after surgery. At 1 month and 3 months after surgery, two pigs remained in grade 3 mitral regurgitation and four pigs

developed grade 4 mitral regurgitation (Figure 2). Table 2 lists the color flow Doppler evaluation of severity of mitral regurgitation of the seven alive pigs at 1-month and 3-month follow-up echocardiographic studies. There was no difference in the vena contracta width, effective regurgitant orifice area, and regurgitation fraction between 1-month and 3-month follow-up echocardiographic studies (Table 2). Of the seven pigs, the parasternal long-axis left atrial diameter increased significantly at 1 month (45.2%, $p = 0.018$) and 3 months (81.1%, $p = 0.018$) after creation of pure mitral regurgitation as compared with baseline values, and the parasternal long-axis left atrial diameter increased significantly at 3 months after surgery as compared with 1 month after surgery (24.8%, $p = 0.018$) (Table 3). In order to clarify whether atrial size enlargement was due to the aging process or not, we compared the atrial size of seven pigs at 3 month after surgery (i.e., 9-month-old) with the atrial size of three 9-month-old control pigs without surgery. Interesting, the mean parasternal long-axis left atrial diameter of the seven pigs at 3 months after surgery (i.e., 9-month-old) was significantly larger than that of the three 9-month-old control pigs (41.3 ± 12.6 vs. 26.6 ± 4.6 mm, $p = 0.033$). Thus, left atrial size enlargement is caused by acute mitral regurgitation and is not due to the aging process. The parasternal long-axis left ventricular end-diastolic diameter increased significantly at 1 month (31.4%, $p = 0.018$) and at 3 months (45.1%, $p = 0.018$) after the creation of pure mitral regurgitation as compared with baseline (Table 3). The parasternal long-axis left ventricular end-systolic diameter increased significantly at 1 month (23.4%, $p = 0.046$) and 3 months (44.4%, $p = 0.028$) after the creation of pure mitral regurgitation as compared with baseline (Table 3). Left ventricular fractional shortening did not change at 1 month and 3 months after the creation of pure mitral regurgitation as compared with baseline (Table 3). The right atrial size did not change at 1 month and 3 months after the creation of pure mitral regurgitation as compared with baseline (Table 3). The mean left atrial areas of the four pigs with grade 4 mitral regurgitation was larger than

that of the three pigs with grade 2 or 3 mitral regurgitation at 1 month after creation of pure mitral regurgitation (14.3 ± 3.8 vs. 10.7 ± 2.9 cm^2, p = 0.289), although the difference did not reach statistical significance. The mean right atrial area of the four pigs with grade 4 mitral regurgitation was larger than that of the three pigs with grade 2 or 3 mitral regurgitation at 1 month after creation of pure mitral regurgitation (11.1 ± 6.0 vs. 9.3 ± 2.6 cm^2, p = 1.000), although the difference did not reach statistical significance. The mean parasternal long-axis left ventricular end-diastolic diameter of the four pigs with grade 4 mitral regurgitation was larger than that of the three pigs with grade 2 or 3 mitral regurgitation at 1 month after creation of pure mitral regurgitation (47.7 ± 7.0 vs. 39.7 ± 7.7 mm, p = 0.157), although the difference did not reach statistical significance. Therefore, atrial and left ventricular sizes had some correlation with grade of mitral regurgitation. Peripheral venous plasma levels of B-type natriuretic peptide did not change at 3 months after the creation of pure mitral regurgitation as compared with baseline (9.7 ± 18.2 vs. 9.3 ± 19.0 pg/mL, p = 0.524).

Three pigs had left atrial samples obtained during surgery before the creation of pure mitral regurgitation and at 3 months after surgery. The long-axis diameters of cardiomyocytes of the left atrial tissues obtained at 3-month follow-up after surgery (18.9 ± 5.5 μm) were larger than the long-axis diameters of cardiomyocytes of the left atrial tissues obtained before surgery (12.8 ± 1.1 μm), although the difference did not reach statistical significance due to small sample size (p = 0.285) (Figure 3). The short-axis diameters of cardiomyocytes of the left atrial tissues obtained at 3-month follow-up after surgery (11.1 ± 2.1 μm) were larger than the short-axis diameters of cardiomyocytes of the left atrial tissues obtained before surgery (8.2 ± 0.8 μm), although the difference did not reach statistical significance (p = 0.109). Additionally, the nuclear sizes of cardiomyocytes of the left atrial tissues obtained at 3-month follow-up after surgery (29.3 ± 8.7μm2)

were larger than the nuclear sizes of cardiomyocytes of the left atrial tissues obtained before surgery (20.9 ± 5.6 μm2), although the difference did not reach statistical significance (p = 0.109). In tissue sections from left atria obtained before surgery, 71.0% of cardiomyocytes had no myolysis, 24.1% of cardiomyocytes had mild myolysis, and 4.9% of cardiomyocytes had moderate-to-severe myolysis. However, in tissue sections from left atria obtained at 3-month follow-up after surgery, 29.0% of cardiomyocytes had no myolysis, 62.9% of cardiomyocytes had mild myolysis, and 8.0% of cardiomyocytes had moderate-to-severe myolysis. Therefore, there were more cardiomyocytes with myolysis after the creation of pure mitral regurgitation as compared with baseline, although the difference did not reach statistical significance (p = 0.109) (Figure 3). The interstitial fibrotic area in the left atrial tissues did not differ between atrial samples obtained before surgery and atrial samples obtained at 3-month follow-up after surgery (0.57 ± 0.46 vs. 0.82 ± 1.07%, p = 1.000).

Table 1. Hemodynamic data of fourteen Lanyu miniature pigs

	Before surgery (n = 14)	After surgery (n = 14)	p Value
LAP (mmHg)	9.7 ± 3.7	12.7 ± 6.3	0.058
RAP (mmHg)	5.2 ± 2.8	5.4 ± 2.9	0.968
PAP (mmHg)	16.9 ± 5.0	18.8 ± 6.4	0.509
SBP (mmHg)	94.4 ± 10.7	89.5 ± 13.8	0.362

LAP: mean left atrial pressure; PAP: mean pulmonary artery pressure; RAP: mean right atrial pressure; SBP: systolic aortic blood pressure.

Table 2. Color flow Doppler evaluation of severity of mitral regurgitation of seven alive pigs

	1-month follow-up	3-month follow-up	p Value
Vena contracta width (cm)	0.69 ± 0.07	0.73 ± 0.11	0.237
Effective regurgitant orifice area (cm^2)	0.37 ± 0.07	0.42 ± 0.13	0.237
Regurgitation fraction (%)	70.3 ± 12.4	61.8 ± 5.0	0.176

Table 3. Echocardiographic data of seven alive pigs

	Baseline	1-Month	3-Month	p Value
LAD (mm)	22.8 ± 5.2	33.1 ± 8.6*	41.3 ± 12.6*†	0.003
LAD index (mm/m^2)	39.1 ± 8.0	54.9 ± 11.5*	62.8 ± 17.1#	0.008
LAA (cm2)	7.9 ± 1.6	12.7 ± 3.7*	16.2 ± 4.3*$	0.002
LAA index (cm2/m2)	17.3 ± 3.0	21.2 ± 6.3*	24.3 ± 5.3**	0.005
RAA (cm2)	8.1 ± 2.5	10.3 ± 4.6	9.9 ± 1.8	0.233
RAA index (cm2/m2)	13.9 ± 4.1	17.0 ± 6.7	14.5 ± 3.0	0.592
LVEDD (mm)	33.7 ± 3.8	44.3 ± 7.9*	48.9 ± 8.3*	0.005
LVEDD index (mm/m^2)	58.0 ± 6.2	71.4 ± 14.5	72.0 ± 15.4	0.076
LVESD (mm)	23.9 ± 2.9	29.5 ± 5.1#	34.5 ± 9.0**	0.027
LVESD index (mm/m^2)	41.4 ± 4.4	49.4 ± 9.5	50.4 ± 15.3	0.101
LVFS	0.29 ± 0.06	0.33 ± 0.05	0.31 ± 0.08	0.481

LAD: left atrial diameter; LAD index : left atrial diameter divided by body surface area; LVEDD: left ventricular end-diastolic diameter; LVEDD index: left ventricular end-diastolic diameter divided by body surface area; LVESD: left ventricular end-systolic diameter; LVESD index: left ventricular end-systolic diameter divided by body surface area; LVFS: left ventricular fractional shortening.
Continuous variables at different time points were analyzed by Kruskal-Wallis test (p Value in the last column)

followed by Wilcoxon signed rank test.

$^{*}p < 0.02$, vs. baseline; $^{\dagger}p < 0.02$ vs. 1-month; $^{\#}p < 0.05$, vs. baseline; $^{\$}p < 0.05$, vs. 1-month; $^{**}p < 0.03$, vs. baseline.

Figure 1. A small hole (white arrow) on the middle scallop of the posterior mitral leaflet has been created.

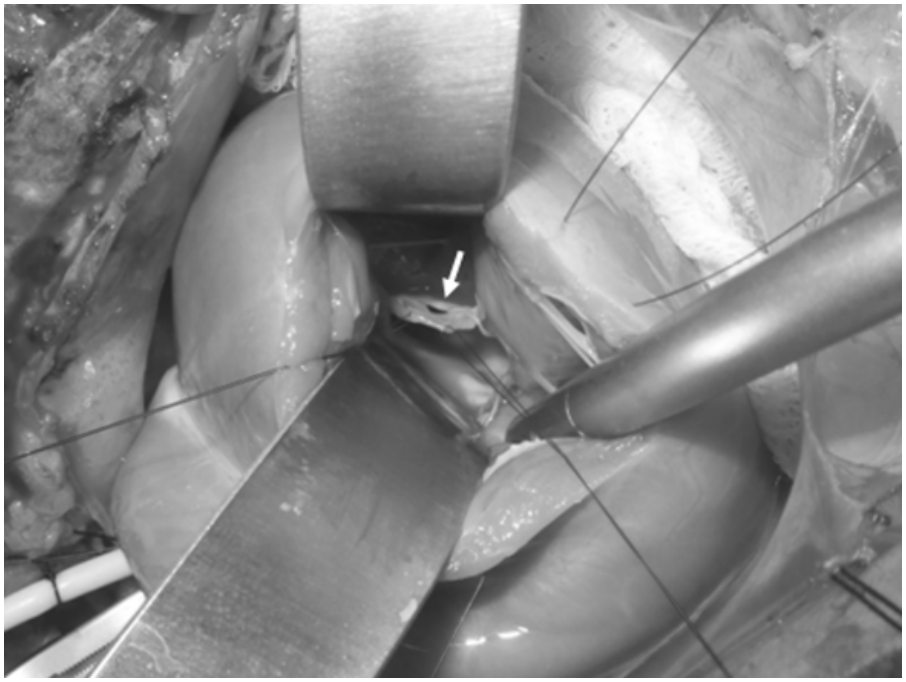


Figure 2. Two-dimensional echocardiographic examinations of 2 pigs at 3 months after surgery showing severe pure mitral regurgitation with eccentric jet on the posterior leaflet of mitral valve. A) modified parasternal long-axis view; B) modified four-chamber view; C,D) color Doppler images.

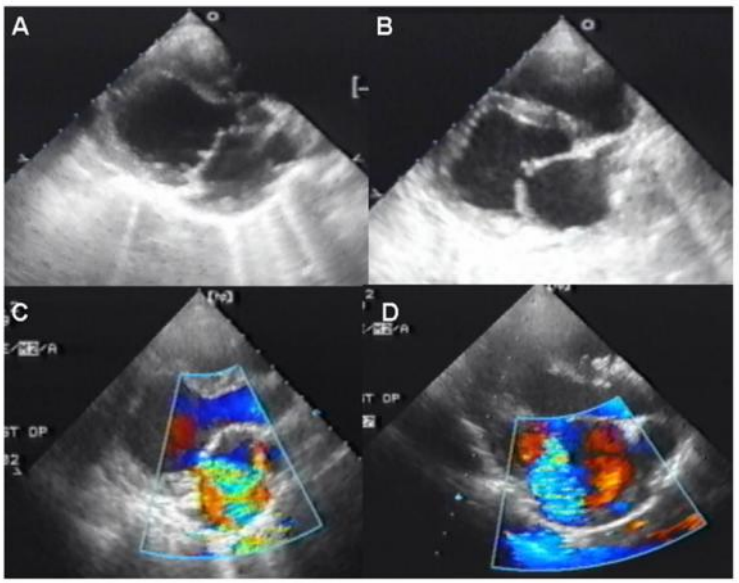
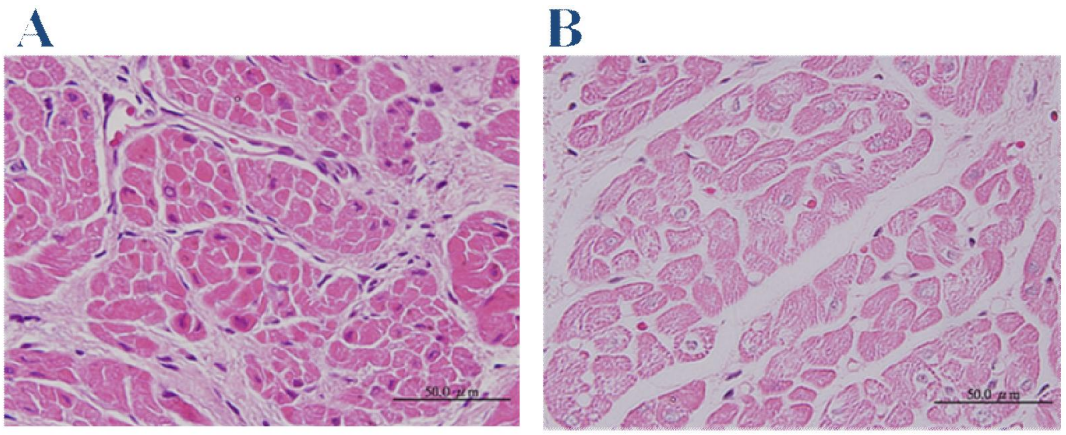


Figure 3. Light microscopical appearance of hematoxylin and eosin stained sections of the left atria of pigs at baseline (A) and at 3-month follow-up after the creation of pure mitral regurgitation (B). The sizes of cardiomyocytes of the left atrial tissues obtained at 3-month follow-up after surgery were larger than the sizes of cardiomyocytes of the left atrial tissues obtained before surgery. There were more cardiomyocytes with perinuclear sarcomere depletion (myolysis) at 3-month follow-up after the creation of pure mitral regurgitation as compared with cardiomyocytes before surgery.



Conclusion

A swine model of pure mitral regurgitation can be successfully established. Left atrial enlargement and structural remodeling develop rapidly and progressively after the creation of significant pure mitral regurgitation.

(2) Not accomplished: Dedifferentation of atrial cardiomyocytes in swine mitral regurgitation may take more than one year to develop, as we observed only some expression of dedifferentation of atrial cardiomyocytes at one-year follow-up.

2.臨床效益：□純基礎研究，無臨床效益
■疾病機轉研究，無立即臨床效益，具臨床意義

□試劑開發，具臨床效益
□疾病診斷，具臨床效益
□藥物療效評估，具臨床效益
□臨床治療與照護評估、具臨床效益
□其他

3.預期可臨床應用之成果: This swine model of pure mitral regurgitation can be used to investigate the molecular mechanisms of myolysis, which may contribute to atrial contractile dysfunction and atrial dilatation in this disease entity.

4.Future plans

We will continue to explore the basic mechanisms responsible for atrial myolysis in significant mitral regurgitation, which is an important cause of heart failure.

5. Acknowledgements

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