

Pathological and Biochemical Analysis of Dilated Cardiomyopathy of Broiler Chickens- An Animal Model

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Abstract

Forty-seven birds (M/F=33/14) with natural outbreak dilated cardiomyopathy (DCM) of right ventricle (RV) and 33 birds with artificially cool-induced DCM hearts were studied. Clinically, 20 severe and 13 mild DCM cases were induced during five weeks and the peak morbidity was in the 2nd week. The progressive dilatation of RV and hypokinesis of septum was shown by echocardiography. At autopsy, the ratio of heart weights to the body weights was increased, the ratio of RV weight to the total ventricle significantly increased, especially in the severe DCM cases ($P<0.05$). The RV was dilated and the wall thickness was increased and finally both RV and left ventricle (LV) were markedly dilated and the septum became thinner. The struts, weave and coil demonstrated by silver impregnation stain were fragmented, dissociated and overstretched. The promatrix metalloproteinases (MMP)-2, -9 and active MMP-2 were markedly increased in natural outbreak DCM cases, especially in the RV ($P<0.05$). The proMMP-2 and active MMP-2 was increased in the cool induced DCM cases, especially in the RV of severe DCM ($P<0.05$). These indicated that both the natural outbreak and the artificially induced DCM of broiler chickens are ideal DCM animal models.

Key Words: cardiomyopathy; extracellular matrix; heart failure; metalloproteinases; ultrasound

Introduction

The ascites syndrome, identified by gross changes of a dilated RV chamber, ascites, congested lungs, shrunken liver and pulmonary hypertension, is a primary cause of death for chickens throughout the world, particularly during a rapidly growing period. It was reported that this syndrome usually happens in three-week old broilers around one week after a cold current during winter (1,5,7-9,12-16,20-22). Many factors, including hypoxia (1,5,16), weight gain (9), feeding diets (7-8,12,14,15), metabolic disturbance

(7), genetic disorder (14,20,21) and other factors (13, 22), are known to induce ascites in broilers. The histological examination by hematoxylin and eosin stain showed significantly different results of chicken with this syndrome, as compared to normal chickens (1,5,7-9,12-16,20-22). In our previous study, 25 myocardial specimens from broilers with naturally occurring ascites syndrome in 3 flocks aged between 21 to 37 days showed DCM in RV (4).

However, the explanation of the pathological findings in RV of broilers remains to be determined. Extracellular collagen matrix, playing an important

role in maintaining normal function of heart, is the most abundant extracellular matrix within the myocardium (11). Extracellular collagen matrix is maintained in a balanced condition in normal heart. However, its level would be altered or decreased in hearts with DCM (19). In addition, the level of matrix metalloproteinases (MMPs) is considered to be the primary factor that is involved in the degradation of extracellular collagen matrix (6). Therefore, the detection of MMP activities and the morphology of extracellular collagen matrix, strut, coil and weave, would be the excellent markers for analyzing pathogenesis of DCM. The pathogenesis of DCM is complicated in human beings. Thus far, human studies focusing on extracellular matrix (ECM) in hearts with DCM are lacking. It is necessary and urgent to set up an animal model to investigate the mechanism by examining the markers described previously. In the previous literatures, the ascites syndrome could be induced by cold-exposure in a short time (1,20-21). By considering the convenience of material preparation, short duration, and ease of control, the broiler could be an ideal animal model to study the pathogenesis of DCM. Accordingly, in the present studies, the natural outbreak and cold-temperature induced DCM hearts of birds were used to investigate the mechanism of ventricular dilatation pathologically and biochemical analysis which was done by using zymography, proteinase assay.

Materials and Methods

All animals were treated and cared for in accordance with the National Institutes of Health "Guide for the care and Use of Laboratory Animals" (NIH Publication No 85-23, revised 1996, National Research Council, Washington, DC, USA).

Experimental Design

Forty seventh (M/F=33/14) Arbor Acres (a line of broiler chickens), aged 37 days, with natural outbreak DCM were collected from 6 different broiler flocks during lowest temperature period in winter in 2000 and 2001. Ten (M/F=4/6) apparently normal chickens were selected as a control group. In the cold-exposure experiment, three-week-old Arbor Acres chickens were treated continuously for 5 weeks with 43 chickens for experimental group and 10 chickens for control group. The temperature was controlled at 14-16°C and 20-25°C for experimental and control groups, respectively. Diets with a metabolizable energy of 3200 Kcal/day were supplied with 20.0% crude protein, 1.2% Arginine and 1.01% Lysine for 3-4 week-old, and with 18.5% crude protein, 0.96% Arginine and 0.94% Lysine for 4-8-week-old.

Food and water were provided ad libitum. Chickens were maintained in a controlled environment with 18h/6h light/dark cycle. Mortality was checked four times per day. Food and water intakes as well as body weights were recorded once per week. Chickens were immediately autopsied if they were found died. Echocardiography was performed using a Sonos 5500 ultrasound machine (Hewlett Packward Co) with 2.5-3.5 MHz phased array transducer for all animals. Hearts were collected for pathological examinations after animals were sacrificed at the end of each week.

Pathological Examinations

The entire heart from each chicken was fixed in 10% neutral-buffered formalin and cut longitudinally to show the four chambers and transversely from the base to the apex serially at 2mm intervals. The length (from the aortic ring to the apex) and width (near the coronary sulcus) of the heart were measured. The total heart weight, left ventricular (LV) weight (including the septum), RV weight, ratio of heart weight to the body weight, the ratio of LV and RV weight to the total heart weight, thickness of left ventricular free wall, septum and RV were measured. Tissue blocks from the LV, septum and RV and those taken from other viscera were embedded in paraffin, sectioned and stained with hematoxylin and eosin and Masson's trichrome blue. For the three types of extracellular collagen matrix (struts, weaves and coils), the silver impregnation stain was used in the sections with 30-35 μ m in thickness (3).

Measurement of MMPs by Zymography

The myocardial samples were prepared for zymographic studies as described previously (2-10). The MMP gelatinase activity and abundance were examined by substrate-specific zymography. The heart samples were homogenized at ice temperature with a model PT10/35 homogenizer for 2 cycles of 10 seconds each. The homogenate was centrifuged at <4°C at 12,000 rpm for 10 minutes. The 40 μ g protein extract was loaded in 1.5 mm of 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel impregnated with gelatin 1 mg/ml. Electrophoresis was run at 150 V for 2.5 h. Enzymes on the gels were renatured by washing twice in a 2.5% Triton X-100 solution with shaking for 30 min. The gels were then incubated with a reaction buffer (50 ml) containing 40 mM Tris-HCl (pH 8.0), 10 mM CaCl_2 , and 0.01% NaN_3 at 37°C for 16 h before staining with 0.25% Coomassie brilliant blue R-250 for 30 min. Quantitative analysis was carried out after discoloring the stain in a discoloring solution (875 ml H_2O , 50 ml methanol, and 75 ml acetic acid). A randomly chosen human breast cancer

Table 1. Right ventricle weight (RV), left ventricle weight with septum (LVS), and ratio of total ventricle, RV, and LVS normalized by BW in 37-day-old male and female chickens with natural outbreak DCM.

Parameters	Males		Females	
	Normal	DCM	Normal	DCM
n	4	10	6	3
RV (gm)	0.99 ^a ±0.11	1.87 ^b ±0.65	0.91 ^a ±0.13	1.96 ^b ±0.14
LVS (gm)	4.37 ±0.26	3.05 ±0.94	3.90 ±0.45	3.60 ±0.71
RV/BW	0.0006 ^a ±0.0001	0.0015 ^b ±0.0005	0.0006 ^a ±0.0001	0.0016 ^b ±0.0003
LVS/BW	0.0025 ±0.0003	0.0024 ±0.0006	0.0024 ±0.0002	0.0029 ±0.0001
TV/BW	0.0031 ^a ±0.0004	0.0039 ^{ab} ±0.0011	0.0030 ^a ±0.0002	0.0045 ^b ±0.0004
RV/TV	0.1842 ^a ±0.0104	0.3778 ^b ±0.0386	0.1882 ^a ±0.0116	0.3557 ^b ±0.0300

^{a-b}: Means±SEM within all variables with no common superscript differ significantly ($P<0.05$)

BW: body weight, HW: heart weight, TV: weight of total ventricle, RV: weight of right ventricle, LVS: weight of left ventricle with septum

Table 2. Length and width of heart, and thickness of right ventricle, septum, and left ventricle at 37 day-old in natural DCM.

Parameters	Males		Females	
	Normal	DCM	Normal	DCM
n	4	20	6	6
Heart length (mm)	28.28 ^{ab} ±0.87	29.80 ^{ab} ±3.64	26.59 ^a ±1.88	30.53 ^b ±1.43
Heart width (mm)	22.63 ^a ±0.96	27.76 ^b ±3.14	22.63 ^a ±0.92	28.12 ^b ±3.69
Thickness (mm)				
RV	1.58 ^a ±0.18	2.63 ^b ±0.54	2.13 ^{ab} ±0.37	2.44 ^b ±0.52
Septum	4.51 ^b ±0.57	2.90 ^a ±0.54	4.03 ^b ±0.44	2.83 ^a ±0.57
LV	4.79 ^b ±0.35	3.36 ^a ±0.48	5.28 ^b ±0.88	3.38 ^a ±0.40

^{a-b}: Means±SEM within all variables with no common superscript differ significantly ($P<0.05$)

RV: Right ventricle, LV: Left ventricle

biopsy extract was used as the marker. Expression of 92 kd (proMMP-9) and 72 kd (proMMP-2) gelatinase in the serum were determined using the Kodak Scientific Imaging Systems SP700 (Eastman Kodak Company, Rochester, NY, USA).

Statistical Analysis

Data are expressed as mean±SEM. One way or 2-way ANOVA or Student's *t* test was used as appropriate. A *P*-value less than 0.05 was considered statistically significant.

Results

Pathological Results of Natural Outbreak Cases

Compared to the normal group, the natural outbreak DCM chickens showed low performance,

generalized congestion, abdominal distension and ascites before they died. At autopsy, massive yellowish to greenish transudates with or without fibrin clots were found in the abdominal cavity. There was moderate amount of pericardial effusion. Lungs demonstrated congestion and edema. Livers were atrophied with vacuolization around central vein of hepatic lobules. Compared with normal chickens of the same age, in both males and females, the body weights were lower and the ratios of heart weight to body weight were higher. The RV weight and ratio of RV to the total heart weight were higher. In contrast, the LV weight and ratio of LV to the total heart weight showed no significant change (Table 1). Interestingly, the heart length, heart width and the thickness of RV were higher, but the thickness of septum and LV were lower (Tables 2). Furthermore, both ventricles were dilated, especially the RV (Fig. 1). There were no significant changes in the four valves, no congenital



Fig. 1. Transverse sections of the normal (lower) and natural outbreak DCM (upper) chicken hearts. Note both ventricles were dilated, especially the RV chamber. The thickness of LV free wall and septum were thinner in DCM heart.

heart disorder such as atrial or ventricular septal defect found. The myocytes showed myocytolysis, weaving and minimal interstitial fibrosis in hematoxylin and eosin and Masson's tircrome blue stain. The extracellular matrix among myocytes including endomysial struts, epimysial wave and perimysial coils decreased and the epimysial waves and perimysial coils were overstretched and fragmented with the silver stain (Fig. 2).

Cold Exposure Experiments

At the time of euthanasia, various degrees of low performance, dyspnea, generalized congestion, abdominal distension and ascites appeared in the experimental chickens as found in natural outbreak cases. Evidence of heart failure was provided by progressive accumulation of pericardial effusion and ascites, progressive dilatation of RV and thinning in thickness and hypokinesis of septum by echocardiography (Fig. 3). Eleven birds died of non-cardiac origins were excluded. The symptoms appeared at week one and the peak morbidity was in the second week. The accumulated morbidity was 47.5% (33/69). They were divided into severe, mild and no DCM if they had marked dilated RV and ascites, without ascites but had pericardial effusion and/or mild dilated RV and had no significant findings at autopsy, respectively (Fig. 4). There were 20 birds with severe DCM and 13 birds with mild DCM. Interestingly, the ratios of heart weight to body weight and the heart width were higher, the thickness of septum and LV were lower, and were directly related to the severity of DCM (Tables 3& 4). In the mild DCM group, myocytes, and the extracellular matrix and other visceral organs showed minimal changes. In the severe DCM group, myocytes, and the extracellular matrix and other viscera organs showed

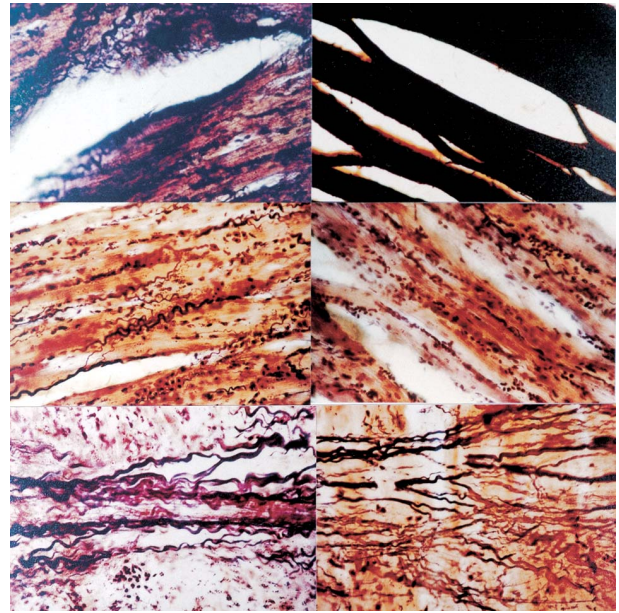


Fig. 2. The extracellular matrix of normal chicken and natural outbreak DCM hearts analyzed by silver impregnation. Upper Right(1): Endomysial struts of normal heart, 400X. Upper Left): Struts were interrupted in DCM heart, 100X. Middle Right): Normal epimysial weaves, 400X. Middle left) Weaves were decreased and dissociated in DCM heart, 400X. Low Right): Normal perimysial coils, 400X. Low Left): Coils were overstretched and fragmented in DCM heart, 400X.

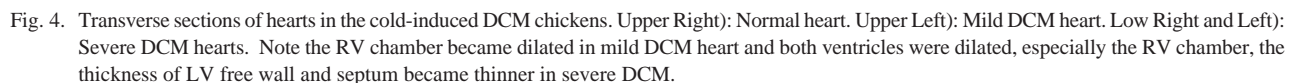
similar findings as those of natural outbreak cases.

Zymographic and Immunoblot Analysis

In natural outbreak DCM cases, there was one band at 92kDa (pro MMP-9) in LV and RV, and the intensity is higher in RV. There were also two other bands appearing at 72kDa and 66kDa that represented pro MMP-2 and active MMP-2, respectively. The intensity of pro MMP-2, -9 and active MMP-2 were markedly increased in LV and RV, with particularly high concentration in RV ($P<0.05$) (Fig. 5). Similar results were present in the cold-temperature induced DCM group (Fig. 6). Interestingly, the intensities of these bands were proportional to the severity of DCM.

Discussion

In the natural outbreak cases, chickens developed severe DCM of RV, with a particularly higher incidence in male (M/F=33/14). The RV weights were increased and became dilated, and it was conjectured that the pathological changes in the lung and liver could be secondary to RV failure (13,22). In the cold-exposure experiment, different stages of the pathological DCM in RV of the experimental chickens were shown. The syndromes appeared at the first



and abdominal sacs. At autopsy, the weights of the hearts were increased, RV chamber dilated and the thickness of septum was increased and both ventricles were dilated. Other organs showed only minimal pathological findings in mild DCM group. There was no evidence of coronary artery disease or myocarditis.

Table 3. Body weight, heart weight, and heart weight to body weight ratio of 56-day-old broilers with DCM induced by cold exposure.

Parameters	Control (20-25°C)	Non DCM (14-16°C)	Mild DCM (14-16°C)	Severe DCM (14-16°C)
n	5	3	3	6
BW (gm)	1480.80 ^a ±116.77	1574.50 ^{ab} ±216.06	1702.25 ^b ±95.81	1234.92 ^a ±225.23
HW (gm)	6.97 ^a ±0.76	9.56 ^{ab} ±1.98	16.03 ^b ±2.89	8.14 ^{ab} ±1.42
HW/BW	0.0047 ^a ±0.003	0.0056 ^{ab} ±0.0007	0.0054 ^{ab} ±0.0007	0.0066 ^b ±0.0006

^{a-b}: Means±SEM within all variables with no common superscript differ significantly ($P<0.05$)

Control: control group, Non DCM: No special gross findings, Mild DCM: Without ascites but hydropericardium or dilated right ventricle in gross findings, Severe DCM: The broilers had ascites in gross findings.

Table 4. Length and width of heart, and thickness of right ventricle, septum, and left ventricle of 56-day-old broilers with DCM induced by cold exposure.

Parameters	Control (20-25°C)	Non DCM (14-16°C)	Mild DCM (14-16°C)	Severe DCM (14-16°C)
n	5	3	3	6
Heart width (mm)	25.95 ^a ±1.96	26.95 ^a ±1.92	29.54 ^{ab} ±4.86	34.87 ^b ±5.41
Thickness (mm)				
RV	1.78 ±0.13	2.04 ±0.53	2.20 ±0.23	2.43 ±0.55
Septum	4.92 ^b ±0.25	4.60 ^{ab} ±0.40	5.00 ^b ±0.28	3.97 ^a ±0.60
LV	6.00 ^b ±0.49	5.85 ^b ±0.50	6.20 ^b ±0.60	4.58 ^a ±0.28

^{a-b}: Means±SEM within all variables with no common superscript differ significantly ($P<0.05$)

RV: Right ventricle, LV: Left ventricle, Severe DCM: The chickens had ascites in gross findings, Control: control group, Non DCM: No special gross findings, Mild DCM: Without ascites but hydropericardium or dilated right ventricle in gross findings.

indicating that all the pathological findings could originate from RV failure. The treated animals gradually developed dilated hearts, a feature very similar to the remodeling of ventricle in human heart failure.

The mechanism must be whereby low temperature induces DCM of chicken is unknown. For example, the cages are overcrowded with chickens and covered with tents resulting in poor ventilation during cold weather. Chickens especially at fast growing period, are almost insufficient to meet oxygen requirements and may easily experience hypoxia (9). In addition, low temperature induces an increased basic metabolic rate and high cardiac output which may cause the conditions of hypoxemia, RV overload and pulmonary hypertension and then progresses into right side heart failure and edema (particular ascites formation) (1,20-21). It has been reported that pulmonary hypertension is considered as the leading cause of ascites in these animals (5,20-21). However some strains that were able to survive chronic pulmonary artery occlusion were more resistant to

ascites induced by cold temperature than the base strains (21). Furthermore, genetic factors (14,20-21), sex difference and metabolic disturbance [7,14], and feeding diets (8,12,15) are reported to affect the ascites syndrome in this animal model. However, no detailed histological studies were performed, nor pulmonary vascular changes are found in these studies, indicating that the pathophysiology do not originate from pulmonary hypertension, nor any of the common causes of congestive heart failure and ascites in chickens (20). Besides, the possible mechanisms might associate with sympathetic activity and hyperthyroidism as well.

The extracellular collagen matrix between myocytes have been identified to play an important role in maintaining the structure and function of the heart. Based on the morphology, these collagen matrices are divided into struts, weaves and coils. The structures of myocardial collagen matrix in non-human primates, such as mice and hamsters, are similar to that of humans. The waves and coils of hearts in pigs with hypertrophic cardiomyopathy are markedly

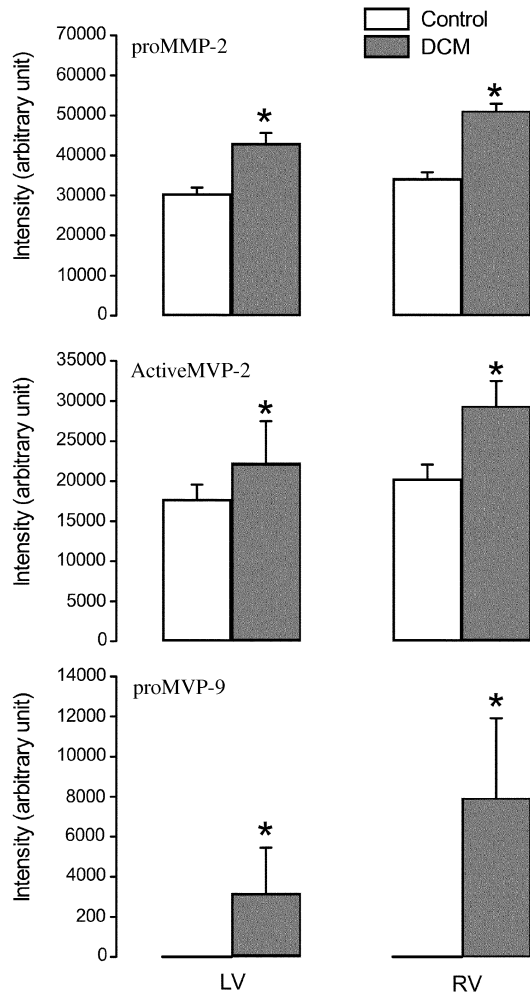


Fig. 5. The intensities of pro-MMP-9, pro-MMP-2, and active MMP-2 of the 37-day-old natural outbreak DCM chicken hearts. Note the intensities were higher ($P < 0.05$) in both ventricles of DCM hearts, especially the RV. Control: control group ($n=6$), DCM: natural outbreak DCM group ($n=6$), LV: left ventricle, RV: right ventricle.

thicker and abundant. The struts are thicker, densely branched, and interconnected, forming a network in the intercellular space (3). In the DCM of chickens, their level of collagen matrix is decreased; collagen matrix is overstretched and fragmented, being similar to those of mammal and human DCM (17-18). This pathological alteration corresponds with the remodeling of ventricles.

The metalloproteinases, MMP-2 (pro-MMP-2, 72kDa; active-MMP-2, 66kDa) and MMP-9 (pro-MMP-9, 92kDa; active-MMP-9, 84 kDa) have been examined in endocardial and subendocardial layers and interstitial tissue. They are involved in the degradation of collagen IV, a major component of basement membrane. In the natural outbreak cases, the pro-MMP-9 activity is increased and consistent with the results of ischemic and non-ischemic human

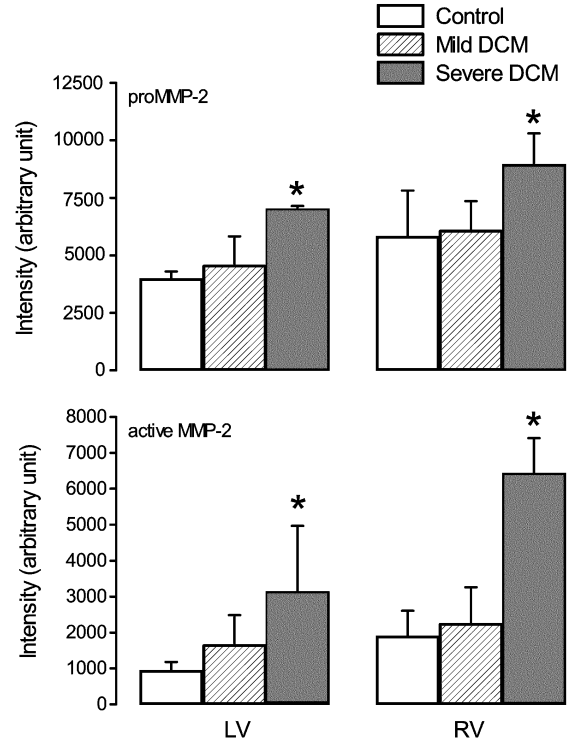


Fig. 6. The intensities of pro-MMP-2 and active MMP-2 of hearts of the 56-day-old cold-induced DCM chicken hearts. Note the intensities were higher in both ventricles of DCM hearts, and the intensity of these bands was in proportional to the severity of DCM. Control: control group ($n=3$), DCM: cold-induced DCM (mild-DCM, $n=3$; severe-DCM, $n=3$), RV: right ventricle, LV: left ventricle.

hearts with DCM. (2,10). In this study, the pro MMP-2 and active MMP-2 were increased in both natural outbreak chickens and cold-exposure chickens. Moreover, the release of MMPs was associated with impaired mechanical function of the heart. These mean that the activities of MMPs were associated with the destruction and lysis of extracellular matrix and then the ventricle became dilated. In conclusion, we have been successfully identified a DCM animal model to study the association of MMP expression and activity with ventricular dilation and congestive heart failure. Further studies including how pharmacological treatments can be helpful in this congestive heart failure animal model are warranted.

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