Investigation of MMP-2 and MMP-9 activities in canine sera with dilated cardiomyopathy

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Summary

Dilated cardiomyopathy (DCM) is accompanied by myocytes and connective tissue changes. Matrix metalloproteinases (MMPs) play important roles in cardiac remodeling. It seems that the gelatinases (MMP-2 and MMP-9) are effective enzymes in cardiomyopathy. Dilated cardiomyopathy was confirmed in 22 dogs (patient group) including 11 female and 11 male by clinical examination, auscultation, thoracic radiography and echocardiography. 17 healthy dogs (control group) with similar weight and breed to patients were also selected from referred cases to Small Animal Hospital of the Veterinary Faculty of Tehran University and the same diagnostic procedures were performed on them. After that, serum MMP-2 and MMP-9 of control and patient groups were measured by semi-quantitative zymography. Semiquantitative analysis of zymograms from canine serums with DCM showed that total MMP-9 in patients is more than control group, while there was no significant difference in total MMP-2 between the two groups. Pro-MMP-2 was not detected in patient group but its active form was present in both groups, of course MMP-2 activity in patients was significantly more than control. Active form of MMP-9 was detected only in patients. Although pro-MMP-9 was present in both groups, its level in control group was significantly higher than patients. The heart enlargement was observed in the left, right or both parts. Statistically significant differences in active form of MMP-2 and MMP-9 levels were observed between different groups of heart enlargement (right, left and both parts) compared to control but this difference was not significant considering chambers affected and VHS (vertebral heart score) groups. In conclusion, although there are some changes in serum MMP-2 and MMP-9 levels in canine DCM, it seems that increase of MMP-9 is more prominent than MMP-2 and neither of them were affected by heart enlargement or VHS grade.

Key words: DCM, Matrix metalloproteinase, MMP-2, MMP-9, Zymography

Introduction

Matrix metalloproteinases are a family of Ca-dependent and Zn-containing endopeptidases; their recognition process started with gelatinase A (MMP-2) discovery in tadpole tails by Gross and Lapiere in 1962. Based on the substrate, characteristics and sequence homology, 25 family members in vertebrates (22 in human) have been described (Yabluchanskiy *et al*., 2013) and classified in 10 subgroups until now (Asawakarn and Asawakarn, 2013). Matrix metallo-proteinase family is one of the most important and effective enzymes in degradation and remodeling of extracellular matrix (Birkedal-Hansen *et al*., 1993). MMPs are involved in some pathological conditions in human such as: arthritis, cardiomyopathy, kerato-conjuctivitis, periodontal diseases, cancer and metastasis (Thomas *et al*., 1998; Baker *et al*., 2002; Asawakarn and Asawakarn, 2013).

One of the most common canine cardiac diseases is acquired myocardial disease and dilated cardiomyopathy (DCM) is the most common form of it. DCM is characterized by an enlargement and impaired systolic function. DCM mostly influences both the left and/or right heart although the left part is more affected than the right side. A variety of myocardial insults including genetic, viral, nutritional and toxic have been mentioned. Although the reason is unknown, somehow DCM is associated with increased volume of the heart (Ettinger and Feldman, 2010).

Fibrillar collagen is the important agent in connecting myocytes and muscle fibers which organize the myocardium (Weber, 1989). The heart activity is the result of coordination between myocyte activities and extracellular matrix (ECM) so the myocardial function, contraction and maintaining heart geometry are dependent on myocardial collagen matrix (Weber, 1989; Thomas *et al*., 1998). Research has consistently shown that extracellular components breakdown and subsequent myocardial remodeling happens in cardiac diseases. However, the myocytes and fibroblasts (Mann and Spinale, 1998) are the main cells to secrete MMPs in cardiac tissue (Galis and Khatri, 2002; Xu *et al*., 2004; Spinale, 2007). Among MMP family, the main substrate

of MMP-2 and MMP-9 is gelatin.

Therefore, it is expected that MMP-2 and MMP-9 changes occur in cardiac diseases. In the other words, the proteins in ECM have an important role in heart function (Senzaki *et al*., 2000). Therefore cardiac enlargement is a result of ECM alteration and finally myocardial collagen matrix changes and it seems that MMP-2 and MMP-9 as gelatinase can be effective in heart size changes.

The potential ability of the MMPs in cardiac disease evaluation is of increasing importance in veterinary cardiology. Although considerable studies exist on MMPs, the majority of them have focused on the cardiac tissue. Sampling cardiac tissue considering possible heart disease of the cases may present a number of problems. In the current research we tried to collect samples from the sera of a common pet dog species (i.e. Terriers) in order to establish a new feasible method of cardiac disease assessment both in sampling and biomarker ability of MMPs. Perhaps understanding accurate cardiac biomarkers can be effective in early diagnosis and treatment. There are some issues about MMPs in human sera but few studies have considered MMPs in canine sera. The aim of our study was to assess the MMPs in sera.

None of the previous researchers have studied the Terrier breed (a medium-size breed). Although some references have suggested that the cardiac disease frequency is higher in this breed (Ettinger and Feldman, 2010), little research has been conducted on this topic. Because of the high prevalence of cardiac disease in Terriers, this breed was suggested for the survey. This assay investigates the different forms of MMP-2 and MMP-9 in the serum of Terrier breed dogs with dilated cardiomyopathy. Investigations of active and inactive forms of MMP-2 and MMP-9 in canine sera with dilated cardiomyopathy and terrier breed have been done for the first time.

Materials and Methods

A total number of 22 dogs including 11 female and 11 male with acquired dilated cardiomyopathy (DCM) were confirmed by clinical examination, auscultation, thoracic radiography and echocardiography. An owner interview including the information about age, sex (intact or neuter), weight, breeds and medical history was performed.

The case breeds were mostly Terrier or a mix of this breed with Poodle, Spitz, Shih tzu, Pekingese, Pomeranian breeds. The average weight and age were 6.40 ± 0.46 kg and 9.59 ± 0.64 year, respectively. The animals with systemic diseases, infection, tumors, diabetes mellitus and inflammation were excluded in this study by medical history, clinical examination and hematology and clinical biochemistry tests. The blood samples were withdrawn from jugular vein immediately upon diagnosis. Serum and plasma were prepared and the samples were stored at -80°C immediately after collection. Additionally, a group of 17 healthy dogs with similar age and weight to patient group were selected from referred cases to Small Animal Teaching Hospital of Faculty of Veterinary Medicine, Tehran University. All examinations that were performed on the patients were done on these cases.

Radiography

Radiographic examination was performed under a standardized protocol with a CR system. High quality lateral and ventrodorsal thoracic projections were taken and cases with radiographic signs of cardiomegaly were included in echocardiographic exam for further evaluation and verification of diagnosis of cardiac disease. These findings were: enlargement of cardiac silhouette relative to the rest of thorax, tracheal and main stem bronchi elevation, increased sternal contact, change in the course of caudal vena cava dorsocranially, the overlapping of the heart and diaphragm in some cases and increased vertebral heart score (VHS) beyond the normal range. In this system, the sum of the apico-basilar length and the craniocaudal maximal width of the cardiac shadow measured at right angles to each other is compared to the length of the vertebral bodies starting at the cranial aspect of T4 (4th thoracic vertebra). The normal range is 8.5-10.5 vertebral bodies (Kealy *et al*., 2010).

Echocardiography

B-mode and M-mode echocardiography were performed by a Vivid 7 echocardiographic machine (General Electric Co. Inc.). Right parasternal short and long axis standard views were made and digitally stored. On B-mode images anatomic structures of the heart, valvular problems, different chamber walls and internal heart structure were scrutinized. On B-mode, right parasternal short axis views the LA:Ao ratio was measured. The M-mode measurements of the left ventricle were obtained by use of standard techniques from a right parasternal short axis view. The M-mode values were used to measure IVSd, LVIDd, LVPWd, IVSs, LVIDs, LVPWs, and fractional shortening. Mitral valve E pointing to septum separation (EPSS) was also measured at the level of mitral valve.

Zymography

Zymography was performed with some adjustments. 15 µL of each serum was diluted by sample buffer (1:1 ratio). 15 µL was subjected to electrophoresis on a 10% SDS-PAGE gel copolymerized with 0.1% gelatin (bovine). Recombinant human MMP-2 (0.1 ng/lane) and MMP-9 (0.2 ng/lane) (Sigma) were loaded on the separated lanes according to the procedure of Laepetit *et al*. (2005). Electrophoresis was done in 20 mA and 96 V under non-reducing condition. The gel was incubated twice, 30 min in Triton-100 X at room temperature and then 24 h in 0.5 M Tris-HCl buffer, pH = 7.4 with 10 mM $CaCl_2$ at 37°C. The gel was stained by Coomassie brilliant blue and de-stained in mixture of acetic acid and methanol. The gels were scanned and analyzed by image analyzer system (my Image Analyzer sotfware). The clear bands against a blue background are accepted as

MMPs. All procedures were performed in duplicate. The 62, 64, 66 and 68-KD forms were accepted as MMP-2 (Coughlan *et al*., 1998; Loukopoulos *et al*., 2003). The 72, 88 and 92-KD were also identified as pro-MMP-2, MMP-9 and pro-MMP-9, respectively (Loukopoulos *et al*., 2003). MMPs levels were estimated by rhMMP-2, -9 and semi-quantitative Zymography (Lepetit *et al*., 2005).

Statistical analysis

Statistical analysis was performed by independent t-student in SPSS package, and results were expressed as Mean ± SE. $P \leq 0.05$ was considered as significant. Also, analysis of variance (ANOVA) and Tukey's Post Hoc tests were used to compare and determine statistical differences in laboratory values between control and patients based on enlargement (complete, left and right side of heart) and VHS groups.

Results

A wide spectrum of clinical signs of DCM were observed in the animals as: varying degrees of depression (100%) and anorexia (100%), dyspnea and tachypnea (81.8%), coughing (63.6%), pulmonary edema (63.6%) and heart murmur (86.3%). Different grades of murmurs were also auscultated ranging from 2/6 to 6/6. Echocardiography revealed generalized (concurrent left and right sided) cardiomegaly in 18.18%, left sided cardiomegaly in 71.73% and right sided cardiomegaly in 9.09% of the cases.

Semiquantitative analysis of zymograms from canine serums with DCM indicates that latent or pro-MMP-2 was not detected in the patient group. The bands related to MMP-2 or active form were present in both groups. The MMP-2 activity in patients was more than control and it was statistically significant while there was no significant difference in total MMP-2 between the two groups.

The level of MMP-9 showed that the active form was only detected in the patients. The pro-MMP-9 was present in both groups but the enzyme level in control group was higher than in patients. There were statistically significant differences in pro, active and total MMP-9 levels (Table 1).

However, no significant changes were observed in serum MMP-2 and MMP-9 levels of control and patient groups with left, right and generalized heart enlargement ($P>0.05$). There were only significant differences between values of MMPs in control and other groups ($P<0.05$) (Table 2).

Survey of MMP-2 and MMP-9 based on VHS determined that there were significant changes between control and groups 1, 2 and 3 ($P<0.05$). On the other hand, the value of pro-MMP-9 was significant between groups 1 and 2 ($P<0.05$) (Table 3). VHS in control group was 8.5-10.5 (normal range).

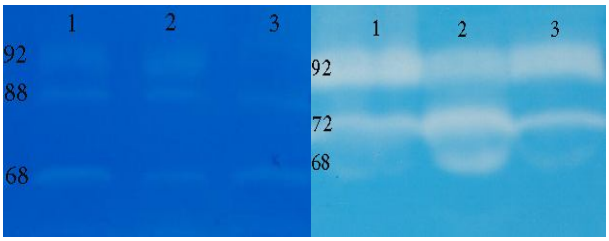

Fig. 1: The left image is related to patient (without 72-KD band) and the right one is normal dogs (without 88-KD band)

Table 1: Serum MMP-2 and MMP-9 levels of dogs with DCM and control group

MMPs	Patient Mean ± SE (Minimum-Maximum)	Control Mean ± SE (Minimum-Maximum)
Total MMP-2	0.64 ± 0.02 (0-0.87)	0.79 ± 0.45 (0-1.69)
Active MMP-2	0.63 ± 0.02* (0.40-0.87)	0.51 ± 0.01* (0.42-0.65)
Pro-MMP-2	0*	0.28 ± 0.07* (0-0.69)
Total MMP-9	1.69 ± 0.16* (0-2.61)	1.087 ± 0.03* (0.9-2.1)
Active MMP-9	1.39 ± 0.04* (0.96-1.96)	0*
Pro-MMP-9	0.28 ± 0.11* (0-1.65)	1.087 ± 0.03* (0.9-1.51)

In each row, asterisk (*) shows significant difference between patient and control group

Table 2: Serum MMP-2 and MMP-9 levels of control and patient groups based on enlargement (left, right and generalized) of the heart

MMPs	Generalized	Left side	Right side	Control
Active MMP-2	0.68 ± 0.06^{b} (0.59-0.87)	0.62 ± 0.02^{b} (0.40-0.83)	0.65 ± 0.13^{b} (0.52-0.79)	0.51 ± 0.01^{a} (0.42-0.65)
Pro-MMP-2	0^{b}	0^{b}	0^{b}	0.28 ± 0.07^{a} (0-0.69)
Active MMP-9	1.40 ± 0.2^{b} (0.96-1.96)	1.37 ± 0.05^{b} (1.01-1.73)	1.48 ± 0.28^{b} (1.2-1.77)	0^{a}
Pro-MMP-9	0.25 ± 0.25^{b} (0-1.01)	0.33 ± 0.15^{b} (0-1.65)	0^{b}	1.08 ± 0.03^{a} (0.9-1.51)
Number	4 (18.18%)	16 (71.73%)	2 (9.09%)	17

Different letters show significant difference ($P<0.05$)

Table 3: Serum MMP-2 and MMP-9 levels of control and patient groups based on VHS

MMPs	Group 1 (VHS 10.6-10.9)	Group 2 (VHS 11-11.9)	Group 3 (VHS 12-13)	Group 4 (control) (VHS 8.5-10.5)
Active MMP-2	0.62 ± 0.06^{b} (0.50-0.81)	0.66 ± 0.02^{b} (0.53-0.79)	0.60 ± 0.06^{b} (0.4-0.87)	0.51 ± 0.01^{a} (0.42-0.65)
Pro-MMP-2	0^{b}	0^{b}	0^{b}	0.28 ± 0.07^{a} (0-0.69)
Active MMP-9	1.50 ± 0.07^{b} (1.29-1.62)	1.39 ± 0.06^{b} (0.96-1.77)	1.33 ± 0.13^{b} (1.01-1.96)	0^{a}
Pro-MMP-9	0^{b}	0.49 ± 0.21^{c} (0-1.65)	0.13 ± 0.13^{bc} (0-0.97)	1.08 ± 0.03^{a} (0.9-1.51)
Number	4 (18.18%)	11 (50%)	7 (31.82%)	17

Different letters show significant difference ($P<0.05$)

Discussion

One of the most common cardiac diseases in the dog is dilated cardiomyopathy. DCM is known by structural hallmarks of cambers dilation, especially left ventricle (Dixon and Spinale, 2009). A prerequisite of dilation is myocytes and ECM changes. Matrix metalloproteinases (MMPs), also called matrixins, function in the extra-cellular environment of cells and degrade both matrix and non-matrix proteins. Besides, the effective role of MMPs in physiological and pathological conditions such as morphogenesis, wound healing, tissue repair, remodeling (Nagase *et al*., 2006), cancer, arthritis and cardiac diseases (Snoek-van Beurden and Von den Hoff, 2005) have been noted. One of the major proteins in the cardiac ECM is collagen and it is the main substrate of MMP-2 and MMP-9. Therefore, it seems that changes in MMP-2 and MMP-9 activities are necessary for chamber dilation. There are several studies in human cardiac tissue and serum that have indicated the changes of MMP-2 and -9 in cardiomyopathies (Bautista-López *et al*., 2013). However, previous studies have noted that pathological and clinical changes in canine cardio-myopathy are attributable to the changes of MMPs in cardiac tissue (Fonfara *et al*., 2013). MMPs secrete as zymogen and will then be activated so there are two types of MMP in the serum or tissues including active or inactive (Latent/pro-MMPs) forms (Van Wart and Birkedal-Hansen, 1990). Accordingly, the present study has been designed to measure MMP-2 and MMP-9 activities in canine sera with DCM by zymography method.

The incidence of DCM in dogs increases with age, usually affecting dogs older than 4 years old (Detweiler *et al*., 1961). The age of patients in our study was 4-15 years and Mean 9.59 ± 0.64. All of the patients had anorexia and depression, but 81.8% and 63.6% of them showed, respectively, dyspnea and coughing with pulmonary edema. As previously mentioned, most of our patients had left side heart enlargement. Generally, dogs with DCM will show evidence of lung problems due to left-sided congestive heart failure, including shortness of breath, rapid, shallow breathing, and coughing. When the ventricles of the heart do not pump enough blood into the lungs, fluid begins to accumulate in the lungs. An enlarged heart soon becomes overloaded, and this often leads to congestive heart failure (CHF) (Ettinger and Feldman, 2010). Papers that have studied the MMP-2 and MMP-9 in cardiac tissue of human and animal with heart failure or DCM, revealed that their enzymes are effective in the disease process and development (Moe and Armstrong, 1999; Dixon and Spinale, 2009).

MMP-2 changes in cardiac diseases have been documented differently in various papers. Increase, decrease and no change have been reported. One of the reasons for different data about MMP-2 is related to sampling time and the etiology of disease. In human, the elevation of MMP-2 is related to the amount of fibrosis in heart tissue (Batlle *et al*., 2007). Because of that, MMP-2 plays a considerable role in human heart diseases that are associated with fibrous tissue enhancement such as infarction. However, ischemia, resulting from reduced blood supply to the heart, activates some inflammatory mediators and MMPs will be increased subsequently. Noji *et al*. (2004) showed that increase in MMPs also occurred in non-ischemic cardiomyopathy and it is similar to non-human species cardiomyopathy. Infarction and ischemia in cardiac tissue in other species except human do not occur frequently (Boon, 2011). As a result, the observed increase in MMP-2 activity in this paper could be attributable to inflammatory process rather than reduced blood supply or fibrous tissue enhancement. In addition, total MMP-2 in our patient group was lower than controls although its active form was statistically higher. Therefore, it seems that the activation process of MMP-2 in patients is more important than enzyme secretion. The findings of the current study are consistent with those of Cheung (Cheung *et al*., 2000). They indicated that there is a large number of pro-MMP-2 molecules in cardiac tissue that will be activated by inflammatory process after injury.

Activation of MMP-2 can impair the heart contractility (Gao *et al*., 2003). MMPs inhibitors reduce dilation (Lindsey *et al*., 2002) and improve heart function (Cheung *et al*., 2000). Therefore, no statistically

significant difference of MMP-2 between patients and control sera could be a compensatory mechanism that inhibits further injuries.

It is interesting to note that in this study, total MMP-9 and active forms are statistically higher in patients. Increase in MMP-9 level is a proven data in different human cardiac diseases (Franz *et al*., 2013). Experimental studies in heart failure have confirmed MMPs elevation over time (Spinale *et al*., 1998). Disease process in experimental studies is totally different with naturally occurred diseases. In natural condition, the process is chronic, but in the experiment, it would be acute. In acute form, ECM degradation is prominent and remodeling mainly happens in the chronic form. However, MMP-9 level in tissue and serum is elevated in ECM degradation (Tousoulis *et al*., 2013) such as naturally occurred diseases but it is reduced in treatment (Wang *et al*., 2013; Yabluchanskiy *et al*., 2013) as the aim of treatment is to reduce ECM degradation. Because of naturally occurring DCM in our study, increase in MMP-9 and activities are not unexpected results.

However, some papers have claimed no significant changes in the serum level of MMP-9, which may be related to the measurement methods. Other research based on antibodies does not distinguish the activity and inactive forms (Kai *et al*., 1998; Baalash *et al*., 2012). We analyzed the active and inactive forms of MMP-2 and MMP-9 by zymography and did not use antibodies.

Several documents revealed that the myocardial and blood level of MMP-9 increase in animal model HF. It may be that the importance of MM-9 is more than MMP-2 but in animal models such as pig and dog, the duration of MMP-2 elevation is longer (Moe and Armstrong, 1999). However, based on our study, it seems that detection of the active form of MMP-9 is more important than active MMP-2 because the active form of MMP-9 was only seen in patients with DCM. In our study, serum MMP-2 and MMP-9 levels of control and patient groups were compared between patients with left, right and generalized heart enlargement and different VHS indices. The results indicated no significant changes between patient groups. However, there was significant difference between pro-MMP-9 in group 1 and 2 of VHS. No relation was detected between the amount of heart enlargement and increase in MMP-2 and MMP-9 levels.

A few previous studies focused on the different (active and inactive) forms of enzymes. Our study revealed the various amounts of different forms of MMP family members in sera. It can therefore be assumed that studying MMPs can be helpful in accurate understanding of cardiac disease etiology and even in finding better biomarkers.

In conclusion, detection of the active form of MMP-9 is more important than active MMP-2 because the active form of MMP-9 was only seen in patients with DCM. It seems that there is potential for using MMPs, especially MMP-9 as a marker in dilated cardiomyopathy. Also, MMP-2 and MMP-9 were not affected by the kind of heart enlargement or grade of VHS. Further studies on the potential cardiac marker characteristics of MMPs are recommended.

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