Renin-angiotensin system enzymes in post-mortem myocardial and renal tissue: a pilot study

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Introduction
Myxomatous mitral valve disease (MMVD) and dilated cardiomyopathy (DCM) are the most common causes of cardiac morbidity and mortality in dogs [1-3]. Activation of the circulating renin-angiotensin-aldosterone system (RAAS) attends to the progression of MMVD [4, 5] and DCM [6, 7]. The RAAS is an important family of enzymes and hormones that regulates fluid and blood pressure homeostasis. In heart disease, the RAAS becomes deranged and promotes disease progression. RAAS blockade with angiotensin-converting enzyme inhibitors (ACE) alone and with spironolactone has been shown to improve survival in dogs with heart failure due to MMVD (ACVIM Stage C) and DCM (MM, 6-18). However, survival is still relatively short even once heart failure develops.

One reason for the ACE-inhibitors may be increased local (tissue) generation of angiotensin II (Ang II) [8]. This peptide is known to be important for disease progression in the cardiovascular system [9, 10], and it is also known for its potential role in the development of organ fibrosis [11]. The local generation of Ang II in the myocardium and kidneys may contribute to the progression of MMVD [4, 5] and DCM [6, 7]. The RAAS is an important enzyme system for the control of blood pressure and cardiovascular functions.

We have sampled 20 dogs so far, 9 of which were sampled according to this objective: determine Ang II formation rate in myocardial and renal tissue samples retrieved within 36 hours post-mortem. The amount of variation in Ang II formation rate and the amount of variation in Ang II formation rate from the same dog seven hours apart differed between individuals. We cannot distinguish changes in Ang II formation rate due to the time difference between sample collection or inter-organ dog variation. The intra-assay coefficient of variation is 10%. An analysis of enzyme stability over time is forthcoming.

The apparent stability of the Ang II/Ang activity ratio over time in each dog suggests that the relative contributions of each enzyme to angiotensin peptide synthesis are reasonably stable over time. The similarity of this ratio between separate sites (LVPW and IVS) suggests that this site is appropriate for RAAS activity analysis. The Ang II/Ang formation ratio is useful to determine the overall balance of (mis)adaptive of the RAAS and may prove to be a helpful biomarker.

Further data analysis will allow us to determine an appropriate sample collection protocol for future studies. Likely, we will aim to collect tissues in a uniform and early (i.e., 4-6 hours) post-mortem window.

Objective 2: determine predominant Ang II-forming enzyme
Surprisingly, ACE, not chymase, appeared to be the predominant Ang II-forming enzyme in the myocardial and renal tissue samples. Ang II was formed at very similar rates in the control and chymostatin trials, suggesting that chymase contributes relatively little to Ang II production in the tissues. In contrast, Ang II formation dropped significantly when isoprenoid was added to the samples. This implies ACE as the major Ang II forming enzyme in the tissues. It also underscores ACE rather than chymase as a target for chronic diseases of these organs.

In contrast, other studies have cited chymase, not ACE, as the major Ang II-forming enzyme in canine heart and kidney [20, 21]. These earlier studies used different assay protocols and different tissue processing techniques, which has been shown to affect enzyme activity [20] and should be avoided. Current methods freeze and preserve the tissue and then perform a homogeneous processing of the sample, which has been shown to improve the rate of Ang II formation.

Other influences on enzyme activity may have affected our results. Although we avoided harvesting major vessels in the tissues, our samples still included small coronary arteries and veins. Angiotensin converting enzyme is bound to cell membranes of endothelial cells, therefore the inclusion of vasculature in our tissue samples could skew our results. Additionally, whereas studies that found chymase dominance in the tissues harvested samples in a matter of minutes post-mortem, we collected our samples 4+ hours after death. Perhaps the post-mortem ischemic environment rapidly changes the relative activity and balance of ACE and chymase in the tissues. We will need to re-evaluate fresh samples to investigate the validity of this hypothesis.

Conclusions
• ACE, chymase, ACE2, and PCP activity can be estimated in samples that are retrieved within 36 hours post-mortem, a realistic timeframe to collect samples from client-owned animals.
• The Ang II/Ang formation rate ratio remained stable between variable 7-hour time intervals post-mortem.
• ACE, not chymase, is the predominant Ang II-forming enzyme in myocardial and renal tissue samples.

Future Directions
We will harvest tissues from pet dogs in heart failure due to naturally-occurring heart disease. Our goal is to collect tissues from dogs in a relatively early and uniform time period post-mortem. We intend to study one group of dogs in heart failure on ACE inhibitors and a second group of dogs in heart failure not on ACE inhibitors. We will perform enzyme activity analyses of myocardial and renal tissue samples from these cases, we will characterize the activity of ACE, chymase, ACE2, PCP, and NEP and describe how ACE inhibitors affect enzyme activity.

Hypotheses
1. ACE, chymase, ACE2, and PCP activity can be measured in samples that are retrieved within 36 hours post-mortem, a realistic timeframe to collect samples from client-owned animals.
2. Chymase, not ACE, will be the predominant Ang II-forming enzyme in myocardial and renal tissue samples.

Methods
At necropsy, 2 g of left ventricular posteri wall (LVWP), 2 g of interventricular septum (IVS), the same level, and 2 g of kidney parenchyma was collected. 2 g of tissue per side (LVWP, IVS, kidney) was immediately frozen at -80 °C and the remaining 1 g of tissue per side was returned to the necropsy cooler (4 °C). Seven hours later, the remaining 1 g of tissue per side was transferred from the necropsy cooler (4 °C) to freezer (-40 °C).

We have sampled 20 dogs so far, 9 of which were sampled according to this study and 5 of which were included in this preliminary analysis (Table 1).

Table 1. Tissue collection times (in hours post-mortem) that were sampled according to this protocol and 5 of which are included in this preliminary analysis (Table 1).

<table>
<thead>
<tr>
<th>Dog</th>
<th>Time (h)</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.5</td>
<td>8.5</td>
<td>6.5</td>
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<tr>
<td>2</td>
<td>14.5</td>
<td>11.5</td>
<td>13.5</td>
</tr>
<tr>
<td>3</td>
<td>13.5</td>
<td>15.5</td>
<td>14.5</td>
</tr>
<tr>
<td>4</td>
<td>22</td>
<td>24.5</td>
<td>26.5</td>
</tr>
<tr>
<td>5</td>
<td>29.5</td>
<td>28.5</td>
<td>29.5</td>
</tr>
</tbody>
</table>

We performed a two-sided t-test to analyze the differences of the means between the groups. We found no significant differences between the groups at the 0.05 level of significance.

Results
Objective 1: detect RAAS enzyme activity
There was measurable activity of ACE, chymase, ACE2, and PCP in myocardial and renal tissue samples retrieved within 36 hours post-mortem. The graphs in Figure 4 display Ang II formation rate over time in myocardial and renal tissue samples from Dogs 1-5. The percentages next to each colored line denote the change in Ang II formation rate between the two time points sampled for each dog. The graphs in Figure 5 depict the ratio of Ang II/Ang II formation rate in the myocardium at both sample collection points for each dog.

Objective 2: determine predominant Ang II-forming enzyme
The scatter dot plots in Figure 6 display the median and range of Ang II formation rate from myocardial and kidney samples incubated with 39P and Moxidizole Ang II (7-36) formation entirely.

Discussion
Objective 1: detect RAAS enzyme activity
ACE, chymase, and PCP activity were successfully estimated in myocardial and renal tissue samples retrieved within 36 hours post-mortem. The amount of variation in Ang II formation rate and the amount of variation in Ang II formation rate from the same dog seven hours apart differed between individuals. We cannot distinguish changes in Ang II formation rate due to the time difference between sample collection or inter-organ dog variation. The intra-assay coefficient of variation is 10%. An analysis of enzyme stability over time is forthcoming.

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