**Evaluation of a novel purine-sensing assay for severity staging of sleep apnea in brachycephalic dogs**

Tingwei Ou and Matthew S. Mellema

UC Davis School of Veterinary Medicine, Davis, CA, USA

**ABSTRACT**

Brachycephalic dogs (BD) with brachycephalic syndrome (BS), such as the English Bulldog, have long been established as an animal model for sleep apnea/hypopnea syndrome (SAHS) in humans. Patients with SAHS oftentimes display excessive daytime sleepiness and suffer from a compromised quality of life. Diagnosis of SAHS in humans is made traditionally by polysomnography and increased morning plasma adenosine levels via High-Performance liquid chromatography (HPLC); however, limitations such as high costs and technical expertise of these procedures have often deterred disease staging. In this study, a commercial adenine detection kit and a novel purine-sensing fluorometric assay that is cost-effective and less labor-intensive were evaluted on their ability to detect differences in morning plasma adenosine concentrations between BD and non-BD. Our principal findings concluded that plasma adenosine concentrations measured with our current homemade fluorometric assay and the BioVision adenine assay kit had no significance between non-BD, Bulldogs, and Boxers. Future endeavors will involve modifying our current homemade assay to further evaluate the disease severity.

**BACKGROUND**

BS is clinically defined as having increased upper airway resistance due to a thickened and elongated soft palate, narrowed nostrils, everted laryngeal sacculae, a hypoplastic trachea, or any combination of the above. Due to these congenital abnormalities, BD with BS are prone to SAHS, where intermittent nocturnal hypoxemia and systemic hypercapnia are commonly observed. Definitive diagnosis of SAHS in BD may be made by measuring hypoxemia markers such as morning plasma/urine purine adenine concentrations via HPLC, which is currently the gold standard for diagnosing SAHS in humans (in combination with polysomnography). However, due to the availability, expense, and technical expertise required for these methods, alternative screening measures have long been sought. English Bulldogs have been established as a spontaneous model for SAHS in humans. Additionally, non-Border-Boxer dogs were shown to have lower PaO2, elevated PCO2, and also elevated arterial blood pressure compared to meso- or dolichocephalic dogs, suggesting that BD share similar pathophysiology with humans with obstructive sleep apnea (2). Boxers, however, appears to be unique among BD breeds in their extremely low incidence of obstructive airway disease. Hence, Boxers would serve as an suitable control within the brachycephalic family.

**SPECIFIC AIMS**

- To determine reference intervals for plasma adenosine in non-BD, Bulldogs, and Boxers.
- To demonstrate whether morning plasma adenosine concentrations are higher in brachycephalic dogs with sleep apnea than those without this condition.
- To determine if our in-house adenosine-sensing assay can be applied to facilitate the diagnosis of sleep apnea in dogs.

**METHODS**

Thirty-two VMTH client-owned dogs including 25 non-BD, 5 bulldogs, and 2 boxers were included in the study after obtaining owners consent. Dogs were considered for the study if they were determined to be in good health, between 1 to 8 years of age, and greater than 55 lbs of body weight. An adenosine stop solution was made to prevent adenosine uptake via red blood cells and deamination via adenosine deaminase during sample collection and storage. Adenosine stop solution consisted of 0.2 mM dipyradiol, 4.2 mM ethylenediaminetetraacetic acid disodium (EDTA), 5 mM 8-mercapto-2-hydroxy-3-nonyl) adenosine (EHNA), 79 μM 5-methyladenosine-5’-diphosphate (AOPCP), heparin sulfate 1 U/mL, and 0.9% NaCl, all of which were purchased from Sigma-Aldrich (1).

Baseline samples were collected either via direct venipuncture of a peripheral vein for the jugular vein. Immediately upon sample collection, 2 mL of blood was injected into 4 mL of ice-cold adenosine stop solution in a 25 mL plastic tube. The sample was inverted several times to achieve adequate mixing, and then split into two commercially available sample collection tubes (“red top”) for immediate centrifugation. Samples were centrifuged at 5000 rpm for 5 minutes. The plasma fraction was then aspirated with a plastic pipette and transferred to a 2 mL, ultra-low temperature compatible storage tube (“cryotube”). Once in cryotube, the sample was immediately stored in the –80°C freezer until further analysis.

Three 96-well microtiter plates were used to sequentially convert purines into hydrogen peroxide and uric acid through the addition of 0.3 U/mL adenosine deaminase (ADA), 0.25 U/mL bacterial purine nucleoside phosphorylase (PNP), and 0.15 U/mL microbial xanthine oxidase (XO) into sample, which will respectively convert purine into adenosine, adenosine into inosine, inosine into hypoxanthine, and hypoxanthine into uric acid and hydrogen peroxide. Hydrogen peroxide was then fluorometrically measured via the addition of 1 U/mL HRP and 10 mM Amplifu red reagent 20 minutes post-incubation at room temperature. The set-up of respective microtiter plates was as follow: one plate included ADA, XO, PNP, and XOP, the last plate only included PNP (P). Potassium phosphate buffer of pH 7.48, 10mM was added in place of the volume of absent enzymes in respective plates. Samples were run in duplicates and read at Ex/Em = 535/585 nm. All reagents above were purchased from Sigma-Aldrich. In addition, a 4th microtiter plate was set up using the BioVision adenine assay kit and read at Ex/Em = 535/585 nm.

A commercially available analysis software package was utilized (Sigmplot 11, Systat Software, San Jose, CA).

**RESULTS**

Plasma adenosine concentrations measured with our current homemade fluorometric assay and the BioVision adenine assay kit had no significance between non-BD, Bulldogs, and Boxers. Further studies are needed to determine the appropriate ADA concentration in our APX reagent required to demonstrate significant differences in plasma adenosine concentrations between BD and non-BD. Additionally, plasma adenosine levels were measured via HPLC to confirm our hypothesis and morning plasma adenosine concentrations are higher in brachycephalic dogs with sleep apnea than those without this condition.

**CONCLUSION**

The principal findings of this study are as follows:

- Plasma adenosine concentrations measured with our current homemade fluorometric assay and the BioVision adenine assay kit had no significance between non-BD, Bulldogs, and Boxers.
- The only commercial adenosine-detecting kit currently available in the market is unsuitable for measuring physiological adenosine concentrations to facilitate the diagnosis of SAHS in dogs.
- The ratio between EHNA concentration in the adenine stop solution and ADA concentration in the APX reagent may be the determining factor in allowing the adenine deamination reaction to proceed in our homemade assay.
- Plasma adenosine levels will be measured via HPLC to confirm our hypothesis and morning plasma adenosine concentrations are higher in brachycephalic dogs with sleep apnea than those without this condition.
- Further study is needed to determine the appropriate ADA concentration in our APX reagent required to demonstrate significant differences in plasma adenosine concentrations between BD and non-BD.
- Ongoing enrollment will expand the sample size for Bulldogs and Boxers to increase statistical power in analyses.

**REFERENCES**


**ACKNOWLEDGEMENT**

Special thanks to Dr. Michael Kent for his amazingly accurate pH meter, and Dr. Chris Murphy for his phenomenal microplate reader, and Michael Katt for his moral support.

**STOP solution contents**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Adenosine (μM)</th>
<th>Adenosine (μM)</th>
<th>Adenosine (μM)</th>
<th>Final plasma (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypotonic saline (control)</td>
<td>17</td>
<td>1.5</td>
<td>0.1</td>
<td>0.24</td>
</tr>
<tr>
<td>Dissolved</td>
<td>11</td>
<td>0.6</td>
<td>0.06</td>
<td>0.13</td>
</tr>
<tr>
<td>EHNA (10 mM)</td>
<td>6.7</td>
<td>0.67</td>
<td>0.06</td>
<td>0.18</td>
</tr>
<tr>
<td>Dissolved</td>
<td>6.7</td>
<td>0.67</td>
<td>0.06</td>
<td>0.18</td>
</tr>
</tbody>
</table>

**Plasma Adenosine (Homemade Assay)**

A) Plasma concentration measured with our homemade fluorometric assay and BioVision adenine assay kit using samples collected from 7 control dogs (non-BD, Bulldogs, and Boxers). No significant differences in plasma adenosine concentration between the control dogs, Bulldogs, and Boxers were noted in either the homemade fluorometric assay or commercial assay.

B) Plasma concentration measured with our homemade fluorometric assay and BioVision adenine assay kit using samples collected from 7 control dogs (non-BD, Bulldogs, and Boxers). No significant differences in plasma adenosine concentration between the control dogs, Bulldogs, and Boxers were noted in either the homemade fluorometric assay or commercial assay.

**Plasma Adenosine (Commercial Assay)**

A) Plasma concentration measured with our homemade fluorometric assay and BioVision adenine assay kit using samples collected from 7 control dogs (non-BD, Bulldogs, and Boxers). No significant differences in plasma adenosine concentration between the control dogs, Bulldogs, and Boxers were noted in either the homemade fluorometric assay or commercial assay.

B) Plasma concentration measured with our homemade fluorometric assay and BioVision adenine assay kit using samples collected from 7 control dogs (non-BD, Bulldogs, and Boxers). No significant differences in plasma adenosine concentration between the control dogs, Bulldogs, and Boxers were noted in either the homemade fluorometric assay or commercial assay.