Short Communication

Activated Partial Thromboplastin Time (APTT) in Dogs Tested by In-House Crude Cephalin from Canine Brain

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Abstract

This research aimed to identify the reference value of activated partial thromboplastin time (APTT) in normal dogs using crude cephalin prepared in-house from canine brain, and to examine its efficacy in detecting disorders of coagulation factors. A total of 105 blood samples were collected from normal dogs between January-May 2010. The APTT value was found to be 10.43-19.08 seconds and significantly correlated with the APTT value of a test using a commercial reagent (Pearson's correlation, r = 0.9923, $p < 0.001$). On testing the reagent's efficacy in detecting disorders of coagulation factors by using cryopoor plasma, it was discovered that the APTT value ranged from 24.50 to 33.44 seconds, and the mean value of the APTT by means of cryopoor plasma was significantly greater than that of normal dogs ($p < 0.001$). The aforementioned APTT value significantly correlated with that obtained by means of a commercial reagent (Pearson's correlation, r = 0.9984, $p < 0.001$). From this research, it can be confirmed that in-house crude cephalin can be substituted for commercial crude cephalin. Besides, the obtained reference range can further be applied in veterinary hematology laboratory.

Keywords: APTT, brain, crude cephalin, dog, reference value

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Introduction

In performing an activated partial thromboplastin time (APTT) test, crude cephalin is used to induce plasma coagulation. Crude cephalin is a form of phospholipids categorized as phosphatidyl ethanolamine, which is found in brain and has characteristics similar to platelet factor-3.

Crude cephalin prepared in-house from canine brain (CEP_{in}) needs uncomplicated preparation and is cost-effective. According to Jaiman et al. (2007), the APTT values obtained by using CEP_{in} significantly correlated with the APTT values obtained by using commercial crude cephalin. In addition, no different result was found among canine brain with different ages, which could then be assumed that CEP_{in} from canine brain can be substituted for commercial crude cephalin. However, the reference APTT value using CEP_{in} from healthy dog were not obtained.

The most coagulation disorders in dogs is Von Willebrand disease (von Willebrand's factor deficiency), and the second-most is Hemophilia A (factor VIII deficiency) (Benjami, 1981; Jain et al., 1993; Day et al., 2000). The APTT value in dogs diagnosed with von Willebrand disease and hemophilia is found to be greater than a range found in healthy dogs (Stokol et al., 1997; Day, 2000; Meyer and Harvey, 2004; DuFort and Matros, 2005; Bauer and Moritz, 2008). In case of limitation in numbers of dogs with coagulation factor deficiencies, cooling of blood plasma (cryoprecipitation) was performed to eliminate coagulation factors, namely von Willebrand factor, factor VIII, XIII and fibrinogen, approximately 40-70% (Lichtman et al., 2006; Hoffman et al., 2008). The obtained cryoprecipitate was an imitation of canine plasma with coagulation factor deficiencies, defined as cryopoor plasma.

The first objective of this research was to identify the reference range of APTT in plasma of normal dogs using CEP_{in}. The second purpose was to determine the diagnosis efficacy of CEP_{in} for the coagulation factor deficiencies in dogs using cryopoor plasma. This research was thus expected to yield the normal reference range of APTT value of the blood tested with CEP_{in} to enable its usage as a substitute for commercial crude cephalin and, subsequently, its commercial development.

Materials and Methods

Sampling: Regardless of sex and breed, 105 dogs aged 1-7 years and weighing over 10 kg between January-May 2010 were used. All dogs were in good health with complete vaccination and anthelmintic treatments. Primary physical examination was initially performed, and only clinically healthy dogs with normal blood profiles were selected. On collecting blood samples, great care was taken while restraining the dogs to ensure as little stress and injury as possible. A signed formal consent form must be obtained from every dog owner.

Preparation of Crude Cephalin from Canine Brain: Three steps in the preparation of CEP_{in} (Jaiman et al., 2007) were as follows: acetone brain extract (ABE), chloroform brain extract (CBE), and preparation of CBE activator mixer (APTT Reagent).

For ABE, all meninges and blood vessels were removed from the brain of dogs died within 12 h and whose causes of death were not related to nervous system. After being cleansed with water, 30 grams of the brain was finely ground with acetone in a mortar, using approximately 150 ml of 0.1 mM butylated hydroxyanisole (BHA) as antioxidant. During this process, the acetone was changed intermittently until transparent. The ground brain was then spread on a plate and let dry in a hood for about 2 h.

In CBE procedure, three grams of the dried ABE was put into a 250 ml Erlenmeyer flask, and 150 ml of 0.1 mM BHA chloroform was added. Using magnetic stirrer and magnetic bar, crude cephalin was extracted for a period of 2 h. Residuum was removed through a 12.5 cm-diameter whatman No. 1 filter paper. To evaporate filtrate, it was transferred to a suction flask and vacuumed until dried up, transforming into dry white residue at the bottom of the flask. Filtrate could also be evaporated by means of cold air. Next, the dry white residue at the bottom of the flask was dissolved with 150 ml of normal saline solution. Two grams of the obtained solution was put into each test tube, and the test tubes were sealed with parafilm and labeled accordingly. After that, the solution was lyophilized until it became dry powder. The lyophilized crude cephalin was then stored at 4°C, in the same manner as commercial crude cephalin's storage.

For preparation of CBE activator mixer, the lyophilized CBE was dissolved in 2 ml of distilled water, then 0.2 ml of the dissolved CBE was mixed and shaken vigorously with 3.8 ml of Michaelis buffer in a 16 x 125 mm flask. Next, 50 mg of kaolin was added, and the flask was sealed with parafilm. The mixture was blended for 2 min, using a vortex mixer, and 6 ml of Michaelis buffer was added and thoroughly blended for another 2 min. This resulted in 10 ml of APTT reagent ready for use.

Collection of Blood Samples: The preparation of plasma samples for APTT test comprised 2 major steps: blood sample collection and plasma preparation in accordance with test objectives. Procedures in collecting blood samples were as follows: approximately 10 ml of each blood sample was taken from either cephalic vein or saphenous vein by means of one syringe method. Invasive blood extraction must not be performed in this regard. Nine ml of blood from the syringe (the first 1 ml of blood that was drawn must not be used) was placed in a plastic test tube together with 3.2% sodium citrate solution, which acted as anti-coagulant. The ratio of the sodium citrate solution to the blood was 1:9. The tube was then sealed, and the combination was carefully mixed to avoid foaming. The blood sample mixture was centrifuged at a speed of 3,500 rpm for 10 min. Plasma was drawn from the mixture, evenly placed in 2 microcentrifuge tubes, and stored at -20°C. These prepared samples were the so-called fresh frozen plasma (FFP).

Experimental design: The obtained FFP was used in 2 separated studies. The first study was to perform APTT

tests in normal dogs to obtain a reference value. The FFP from blood samples was dissolved at room temperature (25°C) and then underwent their APTT tests using CEP_{in} and commercial crude cephalin. The second study was to determine diagnosis efficacy of CEP_{in} for the coagulation factor deficiencies using cryopoor plasma. For preparing cryopoor plasma, the FFP was dissolved at 4°C, and jelly-like precipitates of von Willebrand factor, factor VIII, XIII and fibrinogen were formed at the bottom of the microcentrifuge tube. The substance was then centrifuged at a speed of 3,500 rpm at 4°C for 12 min, and cryopoor plasma was separated afterwards. The cryopoor plasma, imitation of plasma from blood of dogs with coagulation disorders, was then tested for APTT using CEP_{in} and commercial crude cephalin.

APTT tests: APTT tests were performed immediately after the preparation of plasma. For quality control purposes, crude cephalin in the first study was tested with bovine standard plasma and that in the second study with artificial human deficiency plasma prior to the actual APTT tests. Tilt tube technique was applied in the APTT test of each study as follows: 0.1 ml of prepared canine plasma was put in a 12 x 75 mm flask and warmed in water bath at 37°C. Then, 0.1 ml of the solution of CEP_{in} or commercial crude cephalin (C.K. PREST, Diagnostica Stago, Asnieres, France) was mixed in the flask in water bath at 37°C, and stopwatch1 was started simultaneously. After 3 min, 0.1 ml of 0.025 M $CaCl_2$, also kept warm at 37°C, was added, and stopwatch2 was started simultaneously. The flask was tilted at 90 degrees for observation. When clots were visible, the stopwatch was paused and the time was recorded. Each sample was tested in triplicate, and the mean value was calculated accordingly.

Statistical Analysis: The APTT value obtained from the tests under each group was reported in terms of APTT range and mean ± SD. To identify the correlation between the APTT value by CEP_{in} and that by commercial crude cephalin, Pearson's correlation coefficient was calculated using Statistix 8 program and zero intercept option with p-value < 0.05, which is considered as statistically significant. In addition, a paired T-test with p-value < 0.05, which is considered as statistically significant, was applied to investigate differences between the APTT value by CEP_{in} and that by commercial crude cephalin.

Results and Discussion

Of the 105 plasma samples in this research, 48 came from male dogs and 57 from female. One hundred and two dogs were mixed breed, 2 were Golden Retrievers and 1 was a German shepherd. Averages of age and weight were 3.5 years and 16 kg, respectively.

To identify the reference range of the APTT value in normal healthy dogs, initial screening of the blood samples was performed to ensure that the samples came from healthy dogs and regarded as an initial screening in order to detect liver disorders (Prins et al., 2010). The obtained plasma must be free of hemolysis and lipidemia (Moreno and Ginel, 1999) while the value of ALT and ALP must be within a normal range. The range and mean values and standard deviations of the APTT value using CEP_{in} and those using commercial crude cephalin are shown in Table 1. Pearson's correlation analysis showed that the APTT value in the normal dogs' plasma tested using CEP_{in} correlated with that using commercial crude cephalin (rho = 0.9923, $p < 0.001$) (Fig 1). The means and SEM of APTT tested with CEP_{in} and commercial crude cephalin were 13.69 ± 2.09 and 11.96 ± 1.22 seconds, respectively. These values were in the range of normal dog's plasma from previous studies, for example 11.90-21.60 seconds (Moreno and Ginel, 1999) and 17-30 seconds (Benjami, 1981). Kitchen et al. (1996) suggested that the reference value of APTT by a reagent from each company must be established. However, the differences in the APTT values obtained from all the tests must not be greater than 10-20% (Evatt et al., 1992).

Table 1 Ranges, means, and standard deviations of APTT values using in-house crude cephalin from canine brain (CEP_{in}) and commercial crude cephalin (N = 105)

Crude cephalin	APTT range (sec)	Mean ± SD (sec)
CEP_{in}	10.43-19.08	13.69 ± 2.09
Commercial	9.62-15.65	11.96 ± 1.22

Table 2 Ranges, means, and standard deviations of APTT values in cryopoor plasma using in-house crude cephalin from canine brain (CEP_{in}) and commercial crude cephalin (N = 105)

Crude cephalin	APTT range (sec)	Mean ± SD (sec)
CEP_{in}	24.50-33.44	28.71 ± 1.64
Commercial	26.08-30.53	27.97 ± 1.06

As stated above, dogs with von Willebrand disease and Hemophilia A have higher APTT values than the normal range. From our study, the APTT values in cryopoor plasma were significantly greater than that in normal plasma. The APTT range and the mean ± SD in cryopoor plasma tested with CEP_{in} and commercial crude cephalin are demonstrated in Table 2. The correlation coefficient of APTT between testing with CEP_{in} and commercial crude cephalin was 0.9984 ($p < 0.001$) (Fig 2). This finding reaffirms that the result obtained by CEP_{in} is comparable to that obtained by commercial crude cephalin when they are used to

identify a dog's deficiency of coagulation factors, namely von Willebrand factor, factors VIII, XIII and fibrinogen. By using CEP_{in}, the APTT values from cryopoor plasma was higher than that in the untreated plasma ($p < 0.001$) (Fig 3).

From this research, it can be confirmed that in-house crude cephalin can be substituted for commercial crude cephalin. Besides, the obtained reference range can further be applied in veterinary hematology laboratory. With the fact that 90 grams of canine brain yields in-house crude cephalin enough for 1,440 APTT tests (Nantakhruea et al., 2009) and in-house crude cephalin requires uncomplicated preparation as well as is cost-effective, this research reaffirms that crude cephalin prepared in-house from canine brain can well be substituted for commercial crude cephalin in veterinary hematology laboratory.

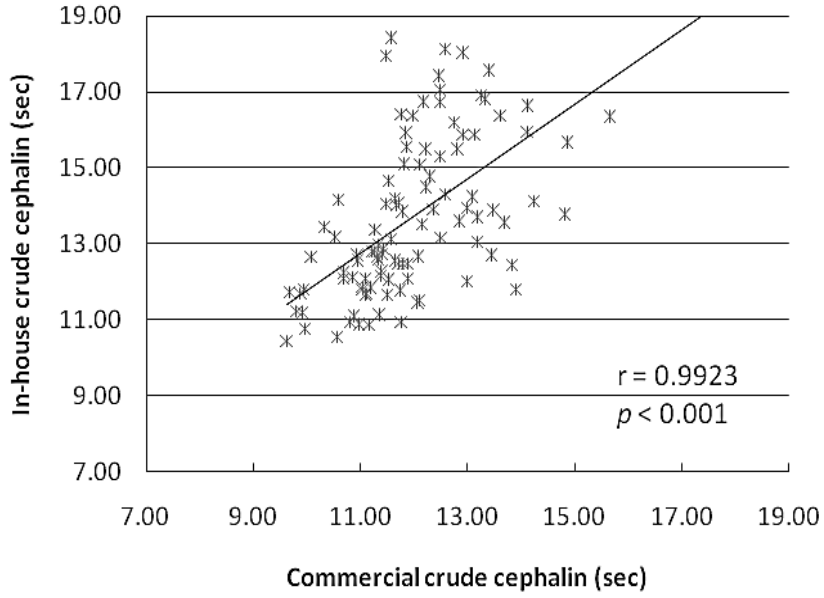

Figure 1 Correlation between APTT values in normal dogs' plasma using in-house crude cephalin from canine brain (CEP_{in}) and commercial crude cephalin

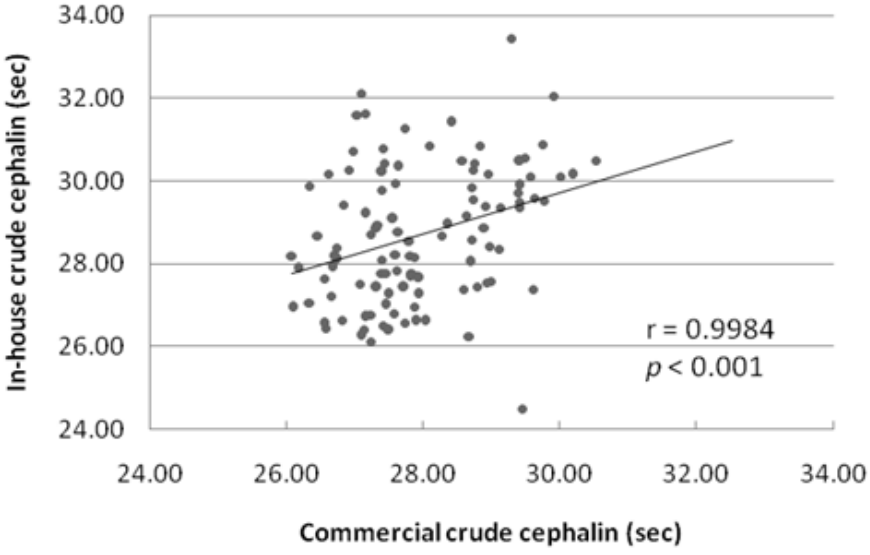

Figure 2 Correlation between APTT values in cryopoor plasma using in-house crude cephalin from canine brain (CEP_{in}) and commercial crude cephalin

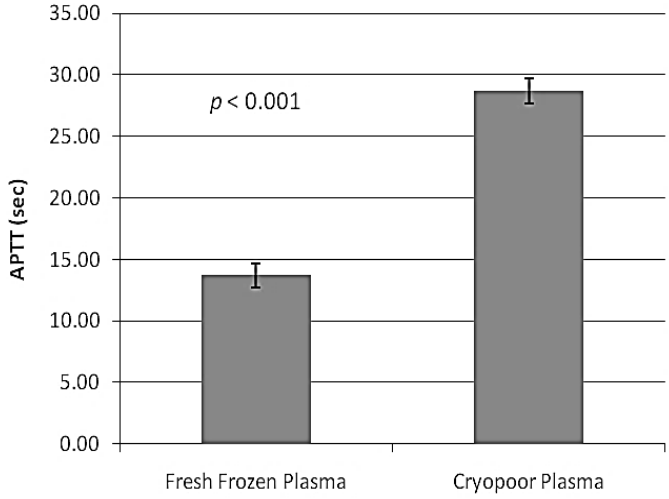

Figure 3 Comparison of APTT value, using in-house crude cephalin (CEP_{in}) and cryopoor plasma, in normal dogs' plasma (fresh frozen plasma).

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บทคัดย่อ

เวลาการกระตุ้นทรอมโบพลาสตินบางส่วนทดสอบโดยใช้เซฟฟารินที่เตรียมจากสมองสุนัข

สราวุฒิ ยะมา[1] แอม ชัยมงคล[1] สมศักดิ์ จันทร์น้อย[2] สาคร พรประเสริฐ[2] วรรณนา สุริยาสถาพร[1]*

วัตถุประสงค์ของงานวิจัยนี้เพื่อหาค่าอ้างอิงของเวลาการกระตุ้นทรอมโบพลาสตินบางส่วน หรือ activated partial thromboplastin time (APTT) ของสุนัขปกติโดยใช้น้ำยา in-house crude cephalin ที่เตรียมจากสมองสุนัข และเพื่อทดสอบประสิทธิภาพที่สามารถใช้หาความผิดปกติของปัจจัยการแข็งตัวของเลือด ทำการเก็บเลือดจากสุนัขปกติ 105 ตัว ระหว่างเดือนมกราคมถึงเดือนพฤษภาคม ปี พ.ศ. 2553 พบว่าค่า APTT เท่ากับ 10.43-19.08 วินาที และพบว่าค่าดังกล่าวมีความสัมพันธ์กันอย่างมีนัยสำคัญกับค่า APTT ที่ได้จากน้ำยาสำเร็จรูป (Pearson's correlation, r = 0.9923, $p < 0.001$) ในการทดสอบประสิทธิภาพของน้ำยาเพื่อหาความผิดปกติของปัจจัยการแข็งตัวของเลือดโดยใช้ cryopoor plasma พบว่าค่า APTT อยู่ในช่วง 24.50-33.44 วินาที โดยค่าเฉลี่ย APTT ของ cryopoor plasma ยาวนานขึ้นกว่าค่าเฉลี่ย APTT ของสุนัขปกติอย่างมีนัยสำคัญ ($p < 0.001$) ซึ่งค่าดังกล่าวกับค่า APTT ที่ได้จากน้ำยาสำเร็จรูปมีความสัมพันธ์กันอย่างมีนัยสำคัญ (Pearson's correlation, r = 0.9984, $p < 0.001$) จากผลการศึกษานี้อาจยืนยันได้ว่า in-house crude cephalin ที่เตรียมจากสมองสุนัขสามารถทดแทนการใช้น้ำยาสำเร็จรูป ค่าอ้างอิงที่ได้สามารถนำไปใช้ในห้องปฏิบัติการทางโลหิตวิทยาทางสัตวแพทย์ต่อไป

คำสำคัญ: APTT สมอง crude cephalin สุนัข ค่าอ้างอิง

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