Original Article

Thrombin Generation Increasing with Age and Decreasing with Use of Heparin Indicated by Calibrated Automated Thrombogram Conducted in Chinese*

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Abstract

Objective Calibrated Automated Thrombogram(CAT) is a test to monitor the generation of thrombin. It can be described by four parameters: lag time, peak thrombin, endogenous thrombin potential (ETP) and time to peak (ttPeak). This study aims to determine the normal ranges of CAT parameters in Chinese, and evaluate whether thrombin generation is correlated with the concentration of heparin/low molecular weight heparin.

Methods Plasma from 120 healthy subjects were collected to determine the normal rangea of CAT parameters in Chinese. Normal plasma pool (NPP, *n*=25) spiked with different concentrations of heparin or enoxaparin were used to detecte CAT parameters. The overall and age specific normal ranges of CAT parameters were calculated using descriptive statistics method with mean±2SD. The correlation between CAT parameters and age or concentrations of heparin, enoxaparin were analyzed with linear regression model.

Results The normal ranges for lag time, peak thrombin, ETP, ttPeak in the subjects were 3.648±2.465 min, 367.39±151.93 nmol/L, 2277±1030 nmol/L·min and 6.372±4.280 min respectively. Age was linearly correlated with lag time (*r*=-0.6583, *P*<0.0001), peak thrombin (*r*=0.4863, *P*<0.0001), ETP (*r*=0.3608, *P*<0.0014) and ttPeak (*r*=-0.6313, *P*<0.0001). The values of ETP/peak ratio were linearly correlated with concentrations of heparin.

Conclusion The normal ranges of four CAT parameters for Chinese were determined. CAT parameters are associated with age. ETP/peak ratio could be used to monitor the process of anticoagulation therapy.

Key words: Calibrated automated thrombogram; Thrombin generation; Age; Heparin; Low molecular weight heparin

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INTRODUCTION

Thrombin plays an important role in thrombosis and hemostasis, it activates factor V, factor VIII, turns over fibrinogen to fibrin that result in formation of clot. Thrombin is important agonist for platelet activation, the generation of thrombin changed under pathological conditions. Thrombin generation decreased under bleeding conditions such as hemophilia, acquired

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pathology anticoagulant[1-2], anticoagulant therapy, for example, heparin using, low molecular heparin using[3-4] etc, and thrombin generation increased under condition of hypercoagulation such as atrial fibrillation[5], venous thromboembolism VTE[6], coronary artery disease CAD[7] and stroke.

The Calibrated Automated Thrombogram (CAT) is a well accepted test for monitoring thrombin generation. The amounts of thrombin were monitored in real time by the intensity of fluorescence released by thrombin substrate. Hemker described CAT as continous determination of potential for thrombin generation in 1993[8]. CAT is a test reflecting global function of coagulation, it shows the balance of coagulation factors and anticoagulation factors. CAT is widely used in research of thrombosis disease[7,9], anticoagulation monitor[10] and bleeding disorder[11]. The reference intervals for various parameters of CAT in Caucasian were determined by Van veen in 2008[12].

Chinese have different blood coagulation-associated genetic background from Caucasian, for example, factor V leiden mutants were frequently observed in Caucasian, but not in Chinese. In this study, we determined the normal range of four CAT parameters and the affected factors, as well as the normal range of CAT parameters in people with different age. In addition, the correlation between ETP/ttPeak and heparin were detected, indicating that the ETP/ttPeak had the potential to monitor anticoagulation treatment.

MATERIALS AND METHODS

Specimens

A total of 120 healthy volunteers (55 males and 65 females), who were fasting, free of medication for at least 2 weeks, informed consent, were enrolled in this study. The average age was 41.43±24.35 years for males and 45.77±19.76 years for females, respectively (Table 1). Questionnaires were used to collected their background information, the results indicated that they had no bleeding disorder, thrombosis, cardiovascular disease, liver, and renal disease. Their blood cell counts, renal and liver function and coagulation including PT, APTT, fibronogen and D dimer were tested and all the parameters were in reference interval. The use of human blood specimens in this study was approved by the Ethical Committee of People's Hospital of Peking University.

Table 1. The age and Gender Distribution of Enrolled Volunteers

Age	Male	Female	Total
<20	11	9	20
20-40	12	18	30
40-60	17	20	37
≥60	15	18	33
Total Number	55	65	120
Average	41.43±24.35	45.77±19.76	

Measurement of CAT Parameters

The measurement was conducted according to the instruction of CAT. Briefly, blood samples were taken and mixed with 129 mmol/L trisodium citrate as 1:9 (v/v) ratio. To obtain platelet-free plasma (PFP), the blood collected was centrifuged twice at 2500 g for 15 min, the supernatant was taken for further use, the platelet concentration were below $10x10^9$/L. Thrombin generations were continuously measured with freshly isolated human PFP. All the samples were tested within 2 h after blood collection. Eighty microliter of PFP was pipetted into the well of a 96-well microplate (Immulon 2HB; Dynex Technologies, Chantilly, VA, USA), then 20 µL mixture of tissue factors (5 pmol/L) and phospholipid vesicles (4.0 µmol/L, phosphatidylserin: phosphatidyl-choline: phosphatidyl-ethanolamine: 1:3:1) were added, which was reconstituted in buffer A (20 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L BSA, pH 7.35). Every specimens were tested in parallel in three wells. Another three wells were parallelly tested by using the same plasma without tissue factor but a fixed amount of thrombin-a2-macroglobulin complex, in order to correct for inner-filter effects and substrate consumption. Plates were inserted into a fluoroskan Ascent well-plate reader (Thermolab Systems, Helsinki, Finland), and preheated at 37 °C for 5 min, the experiments were initiated by automatically adding 20 µL fluorescent substrate-Z-GGR-AMC (2.5 mmol/L), which was dissolved in buffer B (20 mmol/L HEPES, 0.1 mol/L $CaCl_2$, 60 g/L BSA, pH 7.35). After plates were shaken for 10 s, fluorescence generated from cleaved AMC was measured in-time at excitation and emission wavelengths of 390 nm and 460 nm, respectively (37 °C). The intensities of fluorescence were read and the thrombin generation curves were produced by thrombinscope software.

Measurement of CAT Parameters Using Plasma Spiked with Heparin or Low Molecular Weight Heparin

This experiment was performed using normal

plasma pool (NPP). NPP was prepared by mixing the plasma of 25 healthy donors (12 males and 13 females), whose average age was 43.5±15.3 years. Unfraction heparin was from Wuhan biochemical pharmaceutical factory (12 500 U/mL), enoxaparin was from Sanofi-Aventis Clexane (Enoxaparin Sodium Injection) (10 000 U/mL). Heparin and enoxaparin working solution were prepared by diluting the stocks with buffer A to the concentration of 10 anti-Xa U/mL. Then a series of heparin/ enoxaparin spiked NPP plasma were prepared by adding different amounts of working solutions to the final concentration of 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3 anti-Xa U/mL, respectively. CAT parameters were tested by the method described above.

Statistical Analysis

The statistical analyses were performed by using Graphpad 5.0 software (La Jolla, CA, US). The statistical difference in age between males and females was analyzed by *t*-test. CAT parameters between males and females were compared by Mann-Whitney *U* test. The normal ranges of CAT parameters were calculated using descriptive statistics method with mean±2SD. The correlation between age and CAT parameters were analyzed by linear regression model. The normal ranges of CAT parameters for people in different age groups were calculated by descriptive statistics method with mean±2SD. The correlation between CAT parameters and the concentrations of heparin or enoxaparin were evaluated by linear correlation model. $P<0.05$ was considered as statistical difference.

RESULTS

The Normal Ranges of CAT Parameters in Chinese

To get the reference interval of CAT parameters in Chinese, we monitored the individual thrombin generation in 120 persons with enzyme-catalyzed dynamic method. The thrombin generation was shown as a curve, the lag time is the time from reaction onset to the raised up point of the curve, the peak is the highest thrombin generation concentration, the ETP is the area under the curve which represents the potential of total thrombin generation, the ttPeak is the time from onset of reaction to the peak of the curve. The intra-test coefficient of variance of CAT were below 6%, and inter-test coefficient of variance were below 10%. The lag time, peak, ETP, ttPeak showed normal distribution but a relative wide range (Figure 1). The range of them were 2.465-4.825 min, 291.92-441.59 nmol/L, 1794-2840 nmol/L·min and 4.341-8.443 min, respectively (Table 2). These values were close to those of Caucasian, which were 2.47-4.97 min, 325.6-498.6 nmol/L, 1475-2368 nmol/L·min and 5.02-7.53 min, respectively[12], suggesting the difference in genetic background has no effect on the values of CAT parameters.

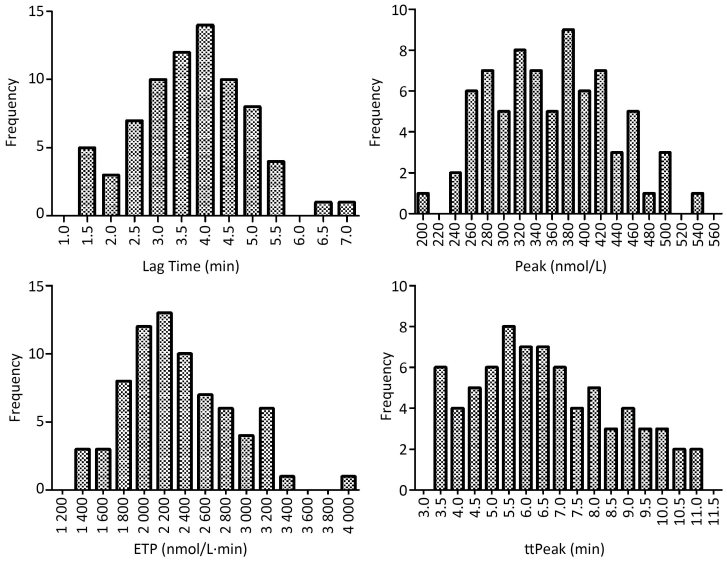

Figure 1. The frequency distribution of lag time, peak thrombin, ETP and ttPeak. X-axis is CATs parameters respectively, Y-axis is the frequency distribution of CAT parameters.

Linear Correlation Between Age and CAT Parameters

The age of volunteers ranged from 3 years to 86 years old, which was in a wide range. To understand the possible effect of age on the values of CAT parameters, we analyzed the correlation between age and CAT parameters by using linear regression model (Figure 2). The sperman *r* of lag time, ttPeak, peak, ETP were -0.6583, -0.6313, 0.4863, 0.3608, respectively. In order to understand whether the *r* was statistical significant, we conducted linear correlation analysis, *P* value of the four parameters were <0.0001, <0.0001, 0.0015, <0.0001, respectively, indicating that the lag time and ttPeak decreased with age, while peak thrombin and ETP increased with age.

The volunteers were 55 males and 65 females, the number was not quite similar. To clarify whether gender would had effect on CAT parameters, we performed following analyses. First, we analyzed the age difference between male group (41.43±24.35 years) and female group (45.77±19.76 years) by using *t*-test. The *P* value was 0.1984, indicating there was no statistical difference. Subsequently, the difference in CAT parameters between males and females was assayed by two-side *t* test. The *P* value of lag time, peak, ETP, ttPeak were 0.6399, 0.0663, 0.0550, 0.1354, respectively, indicating there was no statistical difference. These results demonstrated that gender was not related with the values of four parameters of CAT (Figure 3).

Normal Ranges of CAT Parameters in Different Age Groups

Since the result above demonstrated that age was related with the value of CAT parameters, so it is essential to determin the normal ranges in different age groups. The specimens were divided into four subgroups according to the age of the subjects, i.e. <20, 20-40, 40-60, ≥60 years. The average values for each subgroup were calculated by descriptive statistics method with 95% confidential interval. The normal ranges of CAT parameters in different age groups are listed in Table 3.

Table 2. The Normal Range of CAT Parameters in Chinese

	Minimum	25% Percent	Median	75% Percent	Maximum	Mean±2SD
Lag time (min)	1.330	2.755	3.600	4.470	6.890	3.648±2.465
Peak (nmol/L)	203.6	304.8	371.5	423.1	547.3	367.4±151.9
ETP (nmol/L·min)	1336	1923	2202	2655	3901	2277±1030
ttPeak (min)	3.330	4.745	6.170	7.475	11.220	6.372±4.280

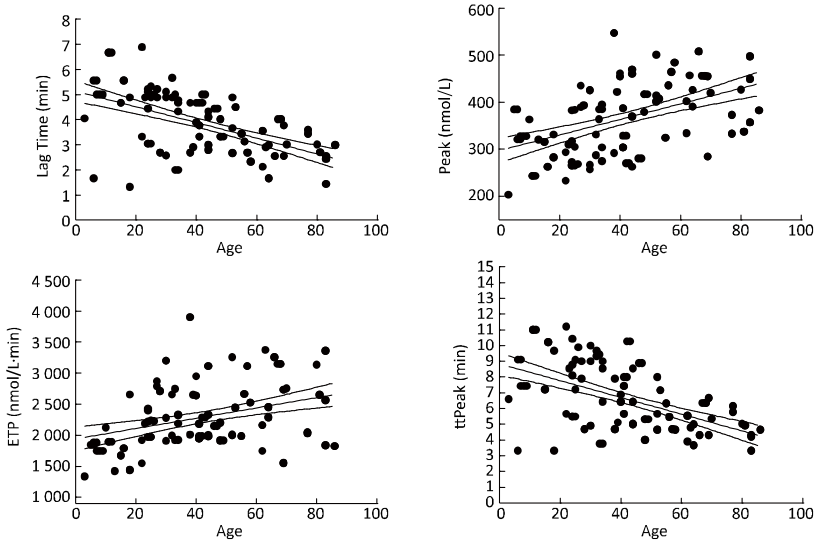

Figure 2. Linear regression of CAT parameters with age. X-axis represents age, Y-axis represents lag time, peak thrombin, ETP, and ttPeak, respectively. Every dot represent one CAT parameter of one specimen. Full lines represent average values of CAT parameters, dotted lines represent 95% confidential interval of these average values.

Correlation between ETP/peak Ratio and Unfraction Heparin Concentration

Heparin and enoxaparin are the most widely used anticoagulation drug. They inhibit thrombin generation in peripheral blood. CAT parameters can be used in monitor thrombin generation, so we used CAT to evaluate the anticoagulation effect of heparin/enoxaparin. As shown in figure 4, the ETP represents area under the curve, which indicates the total potential of thrombin generation. Peak means how high the curve is, suggesting the highest thrombin concentration. ETP/peak ratio can represent mean width of the thrombin generation curve, reflecting the strength of anticoagulation factors. The ETP/peak ratio of normal control group (*n*=120) was 6.279±1.145 min. but the ETP/peak ratio of NPP plasma spiked with heparin was statistically higher compared with normal control group (*P*<0.0001), and linearly correlated with heparin concentration unexpectedly, the ETP/peak ratio of NPP plasma spiked with enoxaparin (6.549±2.784 min) has no significant difference compared with that of normal control (*P*=0.6044). Meanwhile, the curve of Unfraction heparin (UFH) is wider than the curve of low molecular weight heparin (LMWH) at the same anti-factor Xa(anti-Xa) concentration, indicating that UFH can inhibit thrombin generation more efficiently than enoxaparin.

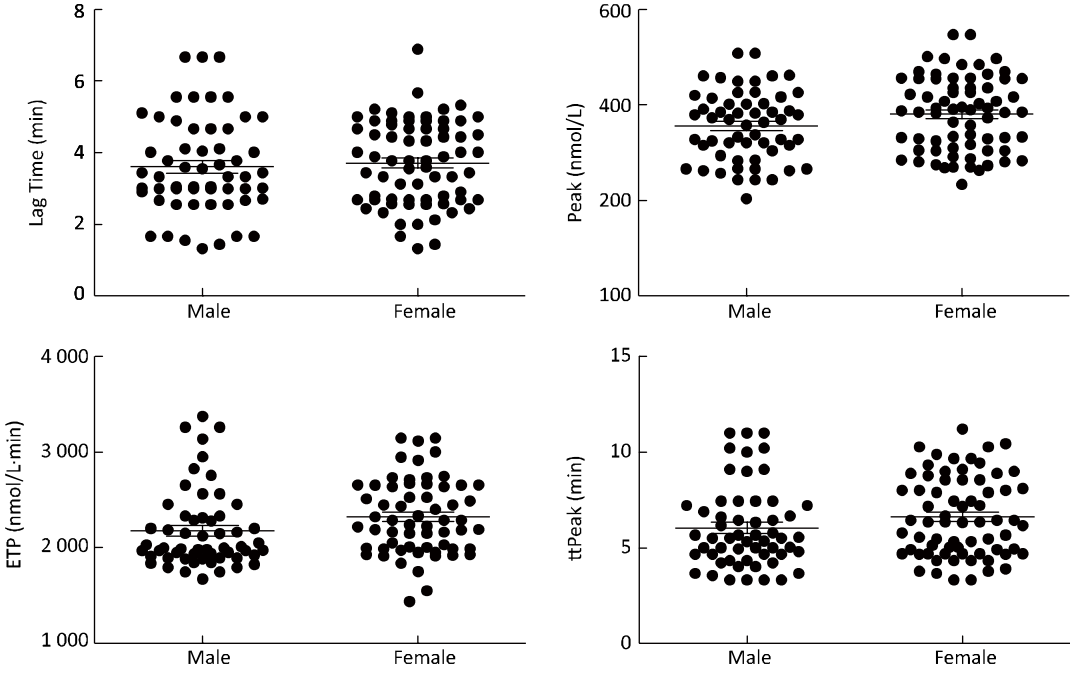

Figure 3. Comparison of CAT parameters between males and females. X-axis represents males and females, Y-axis represents lag time, peak, ETP, and ttPeak, respectively. Every dot represent the value of CAT parameter from one specimen. Long lines represent means of CAT paremeters, short lines represent 95% confidential interval of these means.

Table 3. CAT Parameters Range in Different Groups (show in mean±2SD)

Age	*n*	Lag Time (min)	Peak (nmol/L)	ETP (nmol/L·min)	TtPeak (min)
<20	20	4.908±3.062	303.5±98.0	1787±569	8.210±4.874
20-40	30	4.104±2.412	356.1±165.6	2044±776	7.220±4.268
40-60	37	3.635±1.558	385.8±141.1	2239±805	6.303±3.376
≥60	33	2.902±1.453	406.3±122.9	2485±1150	4.975±1.946
Total	120	3.648±2.465	367.4±151.9	2277±1030	6.372±4.280

DISCUSSION

In this study, we determined the normal ranges for four CAT parameters-lag time, peak thrombin, ETP and ttPeak in Chinese for the first time, and the results showed they were similar with those of Caucasian. We also found the age is linearly correlated with these parameters and the normal ranges in different age groups were determined. It is also reported by Haidl H et al. that the thrombin generation was age-dependent in Caucasion[13]. There were no differences in all these parameters between males and females. The value of ETP/peak ratio is linearly correlated with concentration of heparin in plasma, so it could used to monitor the process of anticoagulation therapy.

Thrombin is an important factor in thrombosis and hemostasis. Its generation can reflect the situation of blood coagulation potential that resulting from balance between prothrombin and antithrombin activity. CAT is a test for measuring thrombin generation, it is standarded by α2-macroglobulin bound thrombin in patients plasma[14]. α2-macroglobulin can bind thrombin to prevent thrombin from being degraded by active proteins in plasma, the bounded thrombin maintain enzyme activity, so it can still cleave substrate to develop fluorescence. This calibrated method detects the exact concentration of thrombin, so it is well accepted up to now[14-16]. Though the test is accurate

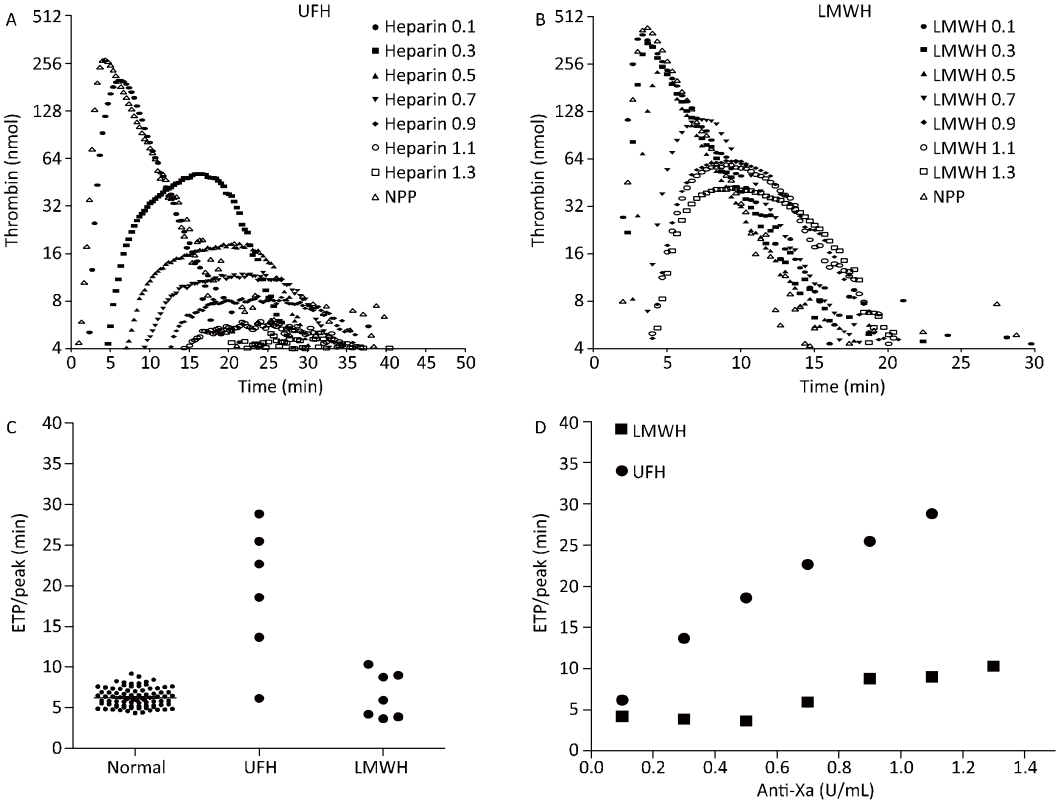

Figure 4. CAT parameters of NPP spiked with UFH/LMWH and comparison of ETP/peak ratio among normal, UFH and LMWH groups. A. CAT curves of NPP plasma spiked with different concentrations of heparin. Each curve represents one titration, X-axis is time of CAT test (min), Y-axis is the thrombin concentration (nmol/L). B. CAT curves of NPP plasma spiked with different concentrations of enoxaparin. Each curve represents one titration, X-axis is time of CAT test (min), Y-axis is the thrombin concentration (nmol/L). C. Comparison of ETP/Peak ratio from normal, heparin and enoxaparin groups, X-axis represents different groups, Y-axis is ETP/peak ratio. Dots represent individual values of ETP/Peak ratio from normal plasma, plasma spiked with heparin or enoxaparin. D. Linear regression analysis of heparin/enoxaparin concentrations and the values of ETP/peak ratio. X-axis represent the concentrations (anti-Xa) of heparin/enoxaparin, Y-axis represents the values of ETP/peak ratio. Dots represent the individual values of ETP/peak ratio in the indicated heparin/enoxaparin titration.

and convenient, we need to establish the reference interval in Chinese before clinical application. As the CAT parameters is linearly correlated with age, the reference interval for the whole population is not reasonable, only the reference interval for different age group can help doctors to estimate the status of coagulation correctly[17]. The samples size was not big in this study, so more samples are needed to determine a exact normal range in the future.

Heparin and enoxaparin are the most widely used anticoagulation drug in hospitals. Activated partial thromboplastin time (APTT) is used to monitor heparin dose and anti-Xa is used to monitor enoxaparin dose. But APTT and anti-Xa are not sensitive enough to reflect the dose of anticoagulation. Also they couldn't really reflect the inhibition effect *in vivo*. CAT directly monitor the thrombin generation, it is more sensitive and more accurate than traditional methods. In our study, the thrombin generation declined from 2700 nmol/L to 300 nmol/L after treated with heparin in a short period of time, but it will take much longer time to reflect by APTT assay, and the risk of bleeding due to overdose could not be avoided. Hence, CAT parameters have potential to substitute the current method in clinical use.

In clinical practice, abnormal CAT parameters may predict further bleeding or thrombosis. CAT parameters can be used in prevention of thrombosis risk in obstetrics, tumor, orthopedic patients[18]. It can also be used in adjusting blood products infusion in bleeding patients such as hemophilia and liver disease patients. The determination of the baseline of CAT parameters in Chinese would make it possible to use CAT in clinical evaluation.

CONFLICT OF INTEREST STATEMENT

The authors disclose that no conflict of interest regarding this manuscript.

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