Chapter 3

METHODOLOGY

Reseach Design : Cross-sectional analytic study in human

Research Methodology : 30 consecutive cases of Green pit viper bite patients are investigated for fibrinolytic parameters. The data will be compared with the other 30 healthy subjects.

Sample Size Justification

The study will compare between 2 groups that are case- and control- group.

In the previous study, t-PA in Green pit viper patients as represented by Euglobulin lysis time (not as accurate and reliable as our methods) is increased in 19 in 51 patients or 37.25% (Sukon Visidhiphan et al, 1989). The pilot study of t-PA level determined by ELISA is not available.

In control subjects, plasminogen activator activity should not be abnormal in more than 5% (95% confidence interval)

For type I error of 5% (one-sided) and type II error of 10% (one-sided),

$$N = [2 (Z_a + Z_b)^2 p q] / (p1 - p2)^2$$

Number of cases in each group should be 28 or approximately 30 cases. The Green pit viper bites that are diagnosed by brought-with snakes is about 30 cases a year in snake bite clinic.

Patient Group

Patients bitten by Green pit viper who come to Snake Bite Clinic, Hematology Unit or who are admitted to general medical ward, Department of Medicine, Chulalongkorn Hospital are eligible for the study. Patients with hematotoxic snake bite who come to OPD is sent to the clinic. Cases with moderate to severe clinical manifestations will be admitted to general medicine ward under the care of hematologists

Inclusion Criteria

Patients with the history of snake bites will be interviewed and examined by the principle investigator or the medical residences who are responsible for snake bite clinic on those days. Patients are enrolled into the study if :

1. Biting snakes are brought with the patients and their species are identified by the main investigator according to Appendix I as Green pit viper.

2. At least one of the following signs of snake bites is demonstrated.

2.1 Fang marks : 2 or more points that look like being injected by needles around bitten areas

2.2 Bullous lesion at the bitten areas

2.3 Skin necrosis at the bitten areas

Exclusion Criteria

Patients are not asked to participate in the study if the following criteria are met:

1. A patient has a history of an underlying disease affecting coagulation and fibrinolytic system such as renal disease, liver disease and congenital coagulation or fibrinolytic defect.

2. Antivenin is administered within 1 month before the study. Indications for antivenin therapy are

2.1 Systemic Bleeding

or 2.2 Unclotted venous clotting time (VCT)

3. Blood or blood product is given within 1 month before the study.

4. A patient took a drug affecting coagulation and fibrinolytic system such as oral anticoagulant, cephalosporin antibiotic and antifibrinolytic drug within 1 week before bite.

5. A patient has at least an organ failure that may cause coagulation or fibrinolytic system activation such as profound shock, severe respiratory distress or severe heart failure.

Control Group

Control subjects are healthy Thai medical personnels who are in the same age group as the patients. They have no underlying disease, no acute illness, no medication and do not exercise at least one hour before their blood are drawn. Their blood will be used for comparing with the fibrinolytic parameters of the patients that are varied between race, age, underlying diseases and different laboratory.

Data Collection

1. History Taking and Physical Examination : Fill in the protocol

Demographic data : age, sex, address, occupation
Underlying diseases, Medication taken
Species of snakes are differentiated according to Appendix 1
Bitten time, Bitten place and Bitten site
Systemic symptoms such as dizziness, vomiting
Time of blood drawn

Local effects	Fang mark distance
	Edema score*
	Ecchymosis
	Blister
	Necrosis
Systemic effect	Bleeding

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* edema score is as followed : 0 = no, 1 = local, 2 = hand/foot, 3 = wrist, ankle, 4 = forearm/leg, 5 = elbow/knee, 6 = arm/thigh, 7 = shoulder/hip and 8 = beyond 7

2. Laboratory Investigations : Methods of blood collection, the variation in fibrinolytic parameter and methods to control it are specified in Appendix II and III

2.1 Automated complete blood count

2.2 Venous clotting time (VCT)

2.3 Complete coagulogram : APTT, PT, TT, Euglobulin lysis time (ELT), Fibrinogen level and Fibrin-fibrinogen degradation products (FDPs)

2.4 Fibrinolytic parameters

Fibrinopeptide A

Plasminogen activity

Antiplasmin activity

t-PA antigen

Plasminogen Activator activity (PA activity)

3. Follow Up Studies

3.1 Outpatient : the patient will be followed every day until 72 hour or more after bite. The local effect, systemic effect, platelet count and VCT are noted.

3.2 Inpatient : the patient who has indication for antivenin therapy will be admitted. The VCT will be done every 6 hour until it becomes less than 30 minutes and once again at 12-24 hour after that time.

The follow-up coagulogram and fibrinolytic parameters will be done on only admitted cases to evaluate the effects of antivenin. The samples will be drawn at the same time of VCT by the processes outlined in Appendix II. The specimens are stored at -80^{o} until the tests are performed.

The technicians who perform laboratory assays will not be informed (blind assessment) about the conditions of the patients.

Outcome Measurements

1. t-PA antigen level is the primary outcome.

2. Plasminogen activator activity reflects either free t-PA level (, if there is t-PA elevation) or direct plasminogen activator activity of the venom (, if t-PA level is normal)

3. Combinations of coagulant and fibrinolytic effect are reflected by APTT, PT, TT, fibrinogen level and FDPs level.

4. Fibrinolytic activation is identified by one of these findings

low plasminogen
low antiplasmin

5. Coagulant effect is measured by fibrinopeptide A antigen level.

Methods

APTT, PT, TT and Euglobulin lysis time	by Conventional methods
Fibrinogen	by Clauss method
FpA (Amiral, Welenga and Fareed,1984)	by ELISA method
t-PA (Holvoet, Cleemput and Collen, 1985)	by ELISA method
Plasminogen (Silverstein, 1975)	by Colorimetric assay
Antiplasmin (Teger-nilsson, Friberger and Gyzander, 1975)	by Colorimetric assay
PA activity (Contant, Thirion and Martinoli, 1987)	by Colorimetric assay

FDPs by Immunoassay

ELISA, Colorimetric assay and Immunoassay kits are purchased from Diagnostica Stago, France

Quality Control

1. 30 healthy control subjects are tested for mean values and standard error of sample mean to compare with manufacturer expected values to determine the accuracy of the tests. The standard deviation and coefficient of variation (CV) are calculated to define the precision of the tests.

2. Pooled plasma samples from 30 healthy subjects are used as control samples in each run. If control value is deviated from expected range, that run will be rejected.

3. Standard control samples for plasminogen and antiplasmin assay are provided by manufacturer.

Interpretation of the Results

The main mechanism of fibrinolysis is

1. t-PA release, if both t-PA and PA activity are elevated.

2. Direct effect of the venom, if t-PA is normal but PA activity is elevated.

3. Secondary to coagulant effect, if t-PA and PA is not elevated but plasminogen is low and correlated with fibrinopeptide A elevation.

Statistical Analysis

1. t-test for means values of each laboratory results in case- and control- group

2. Coefficient of correlation of fibrinopeptide A and fibrinolytic activity

Ethical Consideration

There will be no ethical problem because the blood specimen has to be drawn for VCT assay in routine care. A little more blood is required.

Limitations of the Study

Because this study is cross-sectional, the cause-and-effect relationship is not confirmed. The t- PA level elevations, if there are, may be a cause or a coincidence or even an effect of the hyperfibrinolytic state in Green pit viper bite patients. However, according to the physiological standpoint, t-PA activity elevation should result in hyperfibrinolysis. The other limitation is the comparison group. An ideal control group should be the patients bitten by non-venomous snakes. The proven cases of non-venomous snake bites who come to our hospital are rare. Therefore, We use healthy subjects to be a control group for laboratory methods and physiological variations.