CHAPTER 3

RESEARCH METHODOLOGY

3.1   Research Questions and Objectives

3.1.1 Research Questions

- **Primary research question:**

  Could AGE decrease the incidence of infection of AML patients during remission-induction and consolidation chemotherapy by 30% in comparison to placebo controls?

- **Secondary research questions:**

  1) Was there any difference between the infection related deaths of the two groups?

  2) Did the two groups differ in the total days of fever?

  3) Was there any difference between the two groups in total days of antibiotics given to patients during the period of observation?

  4) Did the two groups differ in the categories of infection namely microbiologically, clinically documented infections or unexplained fever?

  5) Did the two groups differ in fungal colonization?

  6) Was there any difference between the two groups in the proportion of patients to whom antifungal medication was added?

  7) Were there any adverse effects of garlic?

3.1.2 Research Objectives

General objectives

1) To assess the therapeutic effectiveness of garlic in preventing infection of AML patients during chemotherapy.

2) To recommend a simple and easy means of infection prophylaxis for neutropenic patients.

• **Specific objectives**

1) To assess whether oral ingestion of garlic could be tolerated or not.

2) To identify the clinical course of AML patients during and after chemotherapy.

3) To observe fungal colonization of AML patients and see whether the numbers of colonization differed between patients receiving garlic and placebo.

4) To determine the side effect of garlic.

3.1.3 Research Hypothesis

Most infections of chemotherapy induced neutropenic patients were caused by the pathogenic microorganisms colonizing the gastro-intestinal tract. Since garlic is active against those organisms, it might decrease the incidence of infection through alimentary tract decontamination.
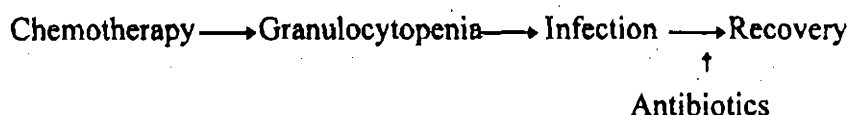
$$H_0: P_T \leq P_C$$

$$H_1: P_T > P_C$$

P_T: The proportion of infection in treatment (garlic) group.

P_C: The proportion of infection in the control (placebo) group.

3.1.4 Conceptual Framework

The mechanisms of infection:

Chemotherapy $\longrightarrow$ Granulocytopenia $\longrightarrow$ Infection $\longrightarrow$ Recovery

$\uparrow$

Antibiotics

Granulocytopenia was the single most important risk factor for infection in patients with acute leukemia. The severity and time period of granulocytopenia was associated with the intensity of chemotherapy. Therefore chemotherapy regimens of the patients were standarized to DA 3+7 (daunorubicin 40mg/m^2/d for 3 days, cytarabine 200mg/d for 7 days) for both the study and the control groups.

The incidences of infection of patients under induction, consolidation chemotherapy and maintenance therapy might be different. Since the infection rate of patients under maintenance chemotherapy was low and the maintenance chemotherapy regimen was different from standard DA 3+7, only newly diagnosed or relapsed AML or AML in consolidation chemotherapy were included in the study. Before randomization, eligible patients were stratified into 3 strata, according to whether they were newly diagnosed, relapsed , or in consolidation chemotherapy.

Because the infection of neutropenic patients was often fatal, empirical broad-spectrum antibiotics were started promptly whenever the patients had fever and infection was suspected. The use of different antibiotics might influence the outcome of the patients with infection or fever. To minimize the bias this might cause, detailed guidelines were used to guide the administration of empirical antibiotics. Staffs were instructed to follow the guidelines strictly.

3.1.5 Operational Definitions

1) Infection: Defined as microbiologically or clinically documented infections, or unexplained fever when the peripheral neutrophil count was < 0.5X10^9/L.

2) Fever: Defined as a single temperature of >= 38.3^{0}C orally in the absence of obvious environmental causes or a temperature of >= 38^{0}C for at least 1 hour.

3) Microbiologically documented infection: Fever episodes accompanied by definite clinical signs and symptoms of infection with microbiological confirmation, defined as bacteremia or a microbiologically defined site of infection (e.g., pneumonia, cellulitis).

4) Clinically documented infection: Defined as fever episodes accompanied by definite clinical signs and symptoms of infection but lacking specific microbiological confirmation.

5) Unexplained fever: Defined as a new fever in the absence of either clinical or microbiological evidence of infection and for which no other causes of fever could be demonstrated.

6) Infection related death: Defined as deaths directly caused by infection, excluding deaths caused by sudden massive bleeding, heart failure or hyperkalemia resulting from tumor lysis.

7) Adverse effect of garlic: Including nausea, vomiting and the severity of bleeding. The severity of bleeding was expressed by the total number of transfusions of platelets and packed red blood cells.

3.2   Research Design

This was a randomized, placebo controlled clinical trial comparing the efficacy of garlic versus placebo in reducing the incidence of infection of AML patients

undergoing remission-induction, reinduction or post-remission consolidation chemotherapy.
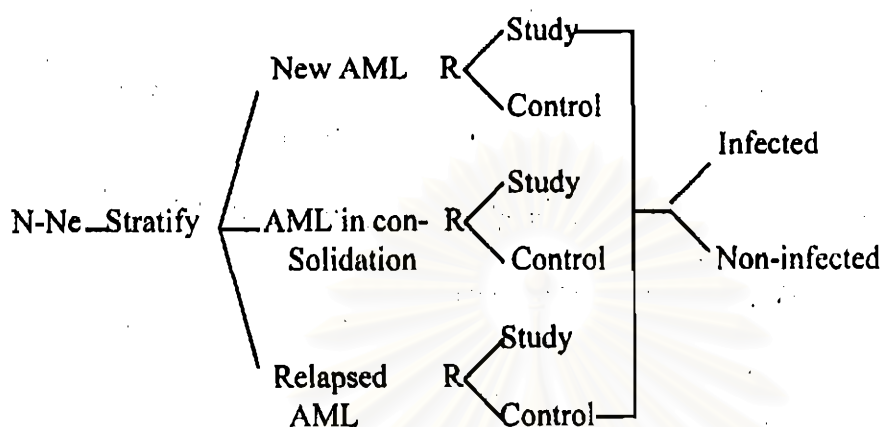
N: All AML patients

Ne: Eligible patients

R: Randomization

3.3 The Sample

3.3.1 Target Population

AML patients undergoing remission-induction, reinduction or post-remission consolidation chemotherapy.

3.3.2 Sample Population

Consecutive AML patients diagnosed at the First General Hospital of West China University of Medical Science who met the eligibility criteria during the research period.

3.3.3 Setting

Hematology ward, First General Hospital, West China University of Medical Sciences, Chengdu, Sichuan, China

3.3.4 Eligibility Criteria

- **Inclusion criteria**

    Table 3.1   Inclusion Criteria

AML patients between years of 15-60 who met one of the following criteria:

- ❖ Newly diagnosed AML
- ❖ AML in remission but undergoing consolidation chemotherapy
- ❖ Relapsed AML undergoing the first course of reinduction chemotherapy

- **Exclusion Criteria**

    Table 3.2   Exclusion Criteria

- ❖ Patients with uncontrolled infection
- ❖ Patients on antibiotics at the time of admission
- ❖ Patients with a history of garlic allergy
- ❖ The very important patients (VIPs) who might not follow the treatment regimen
- ❖ Acute promyelocytic leukemia (M_3) in induction
- ❖ Patients refusing to sign written informed consent

3.3.5 Sample Size Estimation

The primary outcome of the study was the difference between the incidence of infection in patients receiving garlic prophylaxis and placebo control. According to our previous survey, the incidence of infection of AML during chemotherapy was 80% in the ward of hematology of my hospital (unpublished data). Since garlic had

not been used for alimentary tract decontamination before, no data was available as to its effectiveness in controlling infection. After discussion with our hematologists, a difference of 30% was regarded as the effective size which would make a clinical significance.

Sample size was calculated by using the following formula for calculating the sample size for comparing two proportions[27].

$$N = 2[Z\alpha\{2\overline{P}(1-\overline{P})\}^{1/2}+Z\beta\{Pc(1-Pc)+Pi(1-Pi)\}^{1/2}]^2/(Pc-Pi)^2$$

N: The total number of patients of two groups

$Z\alpha = 1.645$, the one-tailed Z value of when $\alpha = 0.05$

$Z\beta = 0.84$, with the power = 80%

Pc: The proportion of infection in the control group, 0.8

Pi: The proportion of infection in the study group, 0.8–0.3 = 0.5

$\overline{P} = (Pc+Pi)/2$

The estimated total sample size was 64, with 32 in each group.

3.4   Experiment Maneuver

3.4.1 Sample Collection

Consecutive sampling was used on AML patients diagnosed at the First General Hospital of West China University of Medical Sciences. Every patient was screened and those meeting the eligibility criteria were enrolled.

3.4.2 Randomization

Block randomization after stratification was applied to this research.

Patients meeting the eligibility criteria were assigned to 3 strata, according to whether they were newly diagnosed, relapsed or in post-remission consolidation.

Within each stratum, patients were then randomized in a block of 2 to receive either garlic or placebo, using the random number table. This stratified randomization helped to make the patients in the two treatment groups homogeneous. The randomization list was generated before the recruitment of the first patient and was kept at the secretary office of the department of hematology, without acknowledgment to the staff. After written informed consent was obtained, the research nurse was given an envelope containing the randomization of the patient. Then from the beginning of chemotherapy, the nurse distributed the medicine to the recruited patient everyday, according to the randomization of the patients.

3.4.3 Blindness

Complete blindness in this study was impossible since garlic had a strong smell. However, measures were taken to minimize the bias which would be otherwise introduced by the difficulty in blindness. The staff and the patients were not informed that this was a placebo controlled study. They were only informed that this study was intended to compare the effectiveness of two kinds of treatment in preventing infection. So doctors' determination in selecting antibiotics for patients with fever would not be influenced by the kind of prophylaxis the patients were receiving.

3.4.4 Intervention

3.4.4.1 Preparation of AGE and Administration

AGE (aqueous garlic extract) was prepared in the pharmacy of my hospital. Garlic cloves were pilled and homogenized in distilled water in a blender for 10 minutes. The suspension was then centrifuged at 5000 r.p.m. for 20 minutes and the supernatant fluid was sterilized by filtering through a 0.22 μm pore size membrane.

The fluid was adjusted by distilled water so that 1 milliliter of final fluid contained the extract of 1 gram of raw garlic. The final fluid was divided into 10 ml aliquots and stored under 4^0C for 5 days. AGE was prepared every 5 days.

There had been no standard dosages of garlic recommended by literature. Studies in China reported using 30-40 grams of raw garlic. Before the start of this study, 6 patients volunteered to take part in a preliminary test, taking AGE 10 ml, 4 times a day. 5 of them complained of severe heart burning on the second or third day. No vomiting was reported by the patients. Then AGE was decreased to 10 ml, 3 times a day on the 5th day and continued for 2 weeks. Only 2 patients complained of slight heart burn which could be well tolerated. Then we decided that the dosage of AGE of this study be 10 ml, 3 times a day, equivalent to 30 grams of raw garlic a day.

Patients of the study group were treated by AGE 10 ml, 3 times a day, starting at the same time of chemotherapy. AGE was continued until 28 days or until the peripheral white blood cell count exceeded $2X10^9/L$ and fever subsided. AGE was taken after meal so as to diminish the irritation to the stomach. The control group were given normal saline by the same dose and schedule.

3.4.4.2 Chemotherapy

Patients of both groups were treated by the same standard DA regimen: Daunorubicin 40 $mg/m^2/d$, intravenous injection in 20 ml of normal saline for 3 days, cytarabine 200 mg/d, continuous intravenous infusion for 7 days.

Concomitant therapy:

Patients in the two treatment groups received the same supportive care to avoid bias.

Ondansetron 8 mg, twice daily, were given intravenously to patients with vomiting during chemotherapy. Transfusions of packed red blood cells were given to patients to maintain the hemoglobin level above 80g/L. Platelet transfusion was given to maintain the platelets above $20X10^9/L$. Patients were transfused with 8-12 units of platelets if their platelets fell below the above level. Examination of the platelet count was performed every two to three days.

All the patients were hospitalized in ordinary ward of the department of hematology during the period of study. Food was prepared by the hospital kitchen and sent to the patients. Fruits were washed with boiled water and peeled before taken by the patients. Medical staffs washed their hands before and after they visited each patient.

3.4.4.3 Management of Patients with Fever and Empirical Antibiotics

Thorough physical examination was performed daily by the attending physician and the residents on the ward. Body temperature of patients was taken every 6 hours or whenever the patients felt unwell. The results of physical examinations and the side effects of the treatment were recorded daily by the residents, then checked and signed by the attending physician. Swab cultures of pharynx and rectum were performed on admission and then once every week to observe the change of fungal colonization.

Complete physical examination and blood culture were performed whenever the patients had fever and infection was suspected. Search was made at the sites most commonly infected. These were periodontium, pharynx, lungs, perineum including anus, skin lesions, bone marrow aspiration sites, vascular catheter exit sites and

fingernails. If there were symptoms or signs of infection of any sites, cultures for bacteria and fungi were performed. If pneumonia or sinusitis were suspected, X-ray films were taken to confirm the diagnosis. Urinalysis was performed and urine was cultured when urinalysis was abnormal or if the patients had symptoms of urinary tract infection. Stool culture was performed for shigellae, salmonellae and fungi if the patients had diarrhea. If the patient did not respond to antibiotic therapy, blood culture and cultures of possible infection sites were repeated every 3 days.

If patients met the criteria of infection, empirical antibiotics were started according to the guidelines below. Those not meeting the criteria were closely observed until infection was diagnosed or until fever subsided. For the few deteriorating patients not meeting the criteria for infection, a hematology specialist group was asked to make a judgment to decide on the initiation of antibiotics.

Antibiotic guidelines:

If the patients met the criteria for infection, antibiotics were started in full dosages immediately after the physical examination and cultures for bacteria and fungi were performed. The initial antibiotic regimens included combinations of an aminoglycoside (amikacin) with antipseudomonal penicillin, piperacillin, or amikacin with a third-generation cephalosporin (cefoperazone or ceftazidime). If patients defervesced within 3 days of treatment, the antibiotics were continued for 7 days and stopped with the peripheral neutrophil count $\geq 0.5 \times 10^9$/L.

If fever persisted >3 days, reassessment on day 4 was done, including a review of all previous culture results, a complete physical examination, reculture of blood and specific sites of infection and diagnostic imaging of any organ suspected of localized

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electrocardiogram, complete blood cell counts, liver and kidney function tests and blood biochemistry tests were performed on admission. Chemotherapy began after the baseline data were collected. Complete blood cell counts and differential counts were performed three times a week to monitor the neutrophil level and the need for transfusion. Bone marrow examination were performed 2 weeks after completion of chemotherapy for assessment of chemotherapy responses.

Outcome measurement

Primary outcome

An independent evaluator who was blinded to the research project decided whether the patients had infection or not, according to the operational definitions. Patients were also considered as infected once the specialist group decided on the initiation of antibiotics.

Secondary outcomes

Infection related death: All the death cases were reviewed by a hematologist group consisting of the residents, attending physicians, professors and the director of the department of hematology to discuss the causes of death. In the very ill leukemic patients, several complications would frequently exist concurrently; however, it would be possible to identify a principal complication leading to the patients' fatal deterioration and sometimes also contributing to secondary complications. Infection related death was considered if infection was the principal complication.

The total days of fever: This was measured according to the temperature record of the patients. Oral temperature of the patients was measured by the nurses on the ward every 6 hours or whenever the patients felt unwell.

The total days of antibiotics given to patients: This was measured according to the case record of every patient. The types of antibiotics and the number of modifications of antibiotics and the addition of antifungal medication were also recorded.

The categories of infection were measured according to the clinical manifestations and the bacterial and fungal cultures.

Fungal colonization was expressed by (1) The proportion of patients with at least one positive fungal colonization culture, (2) The number of positive fungal colonization cultures per patient.

Side effect of garlic

1) Nausea and vomiting: Since chemotherapy would induce apparent nausea and vomiting and ondansetron was given for antiemesis, nausea and vomiting was measured after the completion of chemotherapy. The residents asked the patients whether or not they had nausea and the periods of vomiting in the past 24 hours every morning and recorded them on the case record.

2) Bleeding tendency: Measured by the numbers of transfusions of packed red blood cells and platelets during observation.

Baseline data

Age: The age in years recorded in the identification card.

Sex

FAB subtypes of AML: Measured by morphology and immunocytochemistry study of the bone marrow smears.

Performance status: Measured by using ECOG criteria (see Appendix B).

Hemoglobin, white blood cell count and differential count, platelets on admission, measured by CD-1600K whole blood cell counter.

For AML patients in remission-induction or reinduction chemotherapy, whether or not a complete remission had been achieved was recorded.

The period of severe bone marrow suppression: Expressed by the period of peripheral neutrophil count < $0.5X10^9/L$ (from the completion of chemotherapy to when the peripheral neutrophils => $0.5X10^9/L$).

Bone marrow recovery period: the time period from the completion of chemotherapy to when the peripheral white blood cell count exceeded $2.0X10^9/L$.

3.6   Consideration of Some Confounding Factors

3.6.1 Selection Bias

This was a hospital-based clinical research, which required all eligible patients consulting to the hospital be recruited as the study subjects. To avoid possible missing cases, all the AML patients were screened for eligibility.   Patients accidentally admitted to other departments were referred to the department of hematology.

3.6.2 Assessment Bias

To avoid the bias in assessing the outcomes of the studied subjects, an independent evaluator who was blinded to the study protocol was asked for the assessment. The outcome judgment was according to the operational definitions.

3.6.3. Contamination

Garlic is widely used for cooking in China. The control group might also receive garlic contained in food. However, cooked garlic has no antibiotic activity. In this study, contamination was avoided by requiring patients to take only cooked food.

3.6.4 Co-intervention

Because of the strong smell of garlic, blindness in this study was almost impossible. Co-intervention might occur if attending physicians recognized that the patient was in treatment group by identifying garlic smell and gave extra care, compared to the patients in the control group. Therefore, neither the staff nor the patients were informed about the exact purpose of the study. They were only informed that this study was to compare the effectiveness of two kinds of treatment in preventing infection. Although they could find out that one group was taking a drug containing garlic, they did not know what the other group was taking, which group was the study group. This would help minimize the co-intervention.

Other antibiotics including antifungal medication for infection prophylaxis were not allowed.

3.6.5 Compliance

The compliance in this study was good, since most patients knew that infection was a serious problem. The control group also complied well because they were not informed about using placebo control. The compliance was also ensured by hospitalization of all study subjects, by daily distribution of the medicine and by nurses reminding the patients to take medicine every day.

3.7 Data Collection

The trained residents were responsible for collecting and recording the data of the study on the data record forms (Appendix A). All the variables were recorded according to the operational definitions specified. A research assistant checked whether the data record were correct by interviewing the patients, comparing with the everyday case record and laboratory reports.

3.8 Data Analysis

Intention-to-treat analysis was applied in analyzing the outcome variables. Since all the patients were hospitalized during study, there was no lost to follow up. For the drop out patients, the reasons for drop out and the outcomes of those patients were also reported.

The **baseline data** was expressed as follows:

Table 3.3 Baseline Data

❖ Age	Mean+/-SEM	95% CI
❖ Sex	Proportions	95% CI
❖ FAB subtypes	Proportions	
❖ Performance Status	Proportions	
❖ Hb	Mean+/-SEM	95% CI
❖ WBC	Mean+/-SEM	95% CI
❖ Severe bone marrow suppression	Mean+/-SEM	95% CI
❖ Bone marrow recovery period	Mean+/-SEM	95% CI
❖ Complete Remission	Proportions	95% CI

Primary outcome

The incidence of infection in each group was calculated as the number of patients who were infected divided by the total number of patients enrolled in that group. Comparison of the infection rates of the two groups was computed by Fisher's exact test and the 95% CI for the true difference between the two populations.

Secondary outcomes

Table 3.4   Secondary Outcomes

❖ Infection related death	Proportions	Chi-square test
❖ Total days of fever	Mean+/-SEM	Student's t-test
❖ Total days of antibiotics	Mean+/-SEM	Student's t-test
❖ Types of antibiotics	Proportions	Chi-square test
❖ Numbers of antibiotic modification	Mean+/-SEM	Student's t-test
❖ Categories of infection	Proportions	Chi-square test
◆ Septicemia		
◆ Other Microb. Documented		
◆ Clinical documented		
◆ Unexplained fever		
❖ Fungal colonization		
◆ Proportion of patients with positive surveillance culture	Proportions	Chi-square test
◆ Numbers of positive cultures per patient	Mean+/-SEM	Student's t-test
❖ Addition of antifungal medication	Proportions	Chi-square test
❖ Side effects		
◆ Nausea	Proportions	Chi-square test
◆ Platelet transfusion	Mean+/-SEM	Student's t-test
◆ Packed RBC transfusion	Mean+/-SEM	Student's t-test

3.9   Ethical Considerations

Garlic is widely used in cooking and folk medicine. There is no evidence of myelo-toxicity related to garlic. Although in vitro experiments showed that garlic inhibit the platelet aggregation, there has been no clinical report of garlic induced bleeding. So the most probable side effect would be nausea and vomiting. In our study the AGE was taken after meals to reduce this side effect. A pilot study was carried out to investigate whether 40g/day of raw garlic could be tolerated by the patients. Since most patients complained of severe heart burn, the dosage was decreased to 30g/day.

Since the use of prophylactic antibiotics was not widely accepted in China, most hospitals did not give antibiotics as infection prophylaxis during chemotherapy. So the placebo control would not cause ethical problems.

As mentioned before, this study was difficult to blind. However, if we informed the patients that this was a placebo controlled study, some patients in the control group would take garlic or request for antibiotics, which would introduce tremendous bias into the study. So patients were not informed about using placebo control. Before starting the research, we applied to the ethical committee of my hospital, explaining the reasons for not informing the patients about placebo control. Approval from the ethical committee was obtained.

Patients were completely free to refuse to participate or drop out at any time. Written informed consent was asked from each patient.

3.10   Limitations

Since infected patients were excluded from this study, the generalizability of this study was limited only to non-infected patients.

Autopsy was not accepted by most people in China. So the diagnosis of infection related deaths in this study was not based on postmortem studies.

3.11 Expected Benefits and Applications

Infection is a serious problem in patients with malignant disease and under chemotherapy. This study would provide knowledge of whether a traditional medical plant, garlic could decrease the incidence of infection. The result of the study might help to identify an easy means of infection prevention. Although this study was carried out in AML patients, other neutropenic cancer patients might also benefit from the results of this study.

3.12 Obstacles and Strategies to Solve the Problem

- **Blinding**

Since garlic had a strong smell, this study was difficult to blind. To minimize the bias it might cause, clear criteria for judging the outcomes were given. An independent evaluator who was blinded to the study protocol assessed the primary outcome variable. The detailed proposal of the research was not acknowledged to the staffs on the ward so that they did not know which medicine was the one under study.

- **Patients' compliance**

Patients in this study were all hospitalized so were easy to observe. Nurses distributed the medicine to the patients every morning. The empty bottles were

collected by the research nurses everyday so as to calculate the percentage of the scheduled dosages taken by the patients.

- **Missing and erroneous data**

   This might bias the conclusions. Residents on the ward were trained about the method of patients' management of the study and to also have clear understanding of the operational definitions of the recorded data. The chief investigator checked the completeness and appropriateness of the data.

- **Some particular cases that would not follow the antibiotic guideline**

   Attending physicians were asked to follow the guidelines strictly. Those special patients that would probably refuse to follow the guidelines, such as the close relatives of the attending physicians, very important persons, were excluded from the study.

3.13   Administration and Schedule

Before the start of the research:

- Permission had been obtained from the Ethical Committee of the First General Hospital of West China University of Medical Sciences.

- A meeting was held for the research team and the corresponding medical staff of the Department of Hematology to request their cooperation and participation in the study. Antibiotic guidelines were discussed by the physicians and an agreement to follow strictly the guidelines were reached.

- Two residents were trained about the time for blood examination, bacterial culture, data collection and record according to the operational definitions.

- Preparation of data record forms and written informed consent forms.

This study took one month for preparation, ten months for data collection, one month for data analysis, one month for writing thesis and another month for correction and preparation for final presentation.

Table 3.5 Time Schedule

(April, 1997 to April, 1998)	April	May ---------Dec	Jan	Feb	Mar	April
AGE preparation	—					
Training of personnel	—					
Pilot study for the maximal tolerable dosage of AGE	—					
Data collection		_____				
Data analysis				—		
Thesis writing					___	
Correction and final presentation						___