Establishment of an Aplastic Anemia Model in New Zealand Rabbits (Post-print)

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Abstract

Objective: To screen for the appropriate dosage of benzene and cyclophosphamide in combination for establishing an aplastic anemia model in New Zealand rabbits using orthogonal design. Methods: New Zealand rabbits were subjected to orthogonal experimental design, employing an L9(3^4) orthogonal array for four factors—benzene dosage (A), cyclophosphamide dosage (B), benzene injection frequency (C), and cyclophosphamide injection frequency (D)—each at three distinct levels, to establish an aplastic anemia model in New Zealand rabbits, ultimately selecting an optimal modeling protocol from nine experimental groups. The administration method involved subcutaneous injection of benzene on the back every other day, followed by intravenous injection of cyclophosphamide via the ear margin daily, both administered according to specified frequencies. Complete blood count (CBC) was performed once every 6 days, and a small segment of femur was taken for bone marrow histological examination before modeling and on day 36 post-modeling to observe changes. Results: Comparative analysis of white blood cell, red blood cell, and platelet counts among the nine groups revealed successful establishment of the aplastic anemia model in New Zealand rabbits in groups 4-9. The difference in average daily decline rate between groups 7, 8, and 9 and the other groups was statistically significant ($P<0.05$). Bone marrow sections from group 7 showed bone marrow hypoplasia, reduced hematopoietic tissue, decreased or absent megakaryocytes, and increased adipocytes. Follow-up revealed persistent bone marrow suppression in group 7, consistent with the clinical characteristics of this disease. Conclusion: Group 7 employed benzene 1.5 mL/kg, 8 times/day, and cyclophosphamide 10 mg/kg, 4 times/day. This combination dosage established a New Zealand rabbit AA model with a short modeling period and stable model, representing a modeling method that can be widely applied in animal experiments.

Full Text

Preamble

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Abstract

Objective: To screen for the optimal dose of benzene and cyclophosphamide using an orthogonal design for establishing New Zealand rabbit models of aplastic anemia. **Methods:** Following an orthogonal experimental design, we tested three levels of four factors—namely the dose of benzene (A), the dose of cyclophosphamide (B), the number of benzene injections (C), and the number of cyclophosphamide injections (D)—in the establishment of New Zealand rabbit models of aplastic anemia using an L9 (3^4) orthogonal table. The optimal protocol was selected from nine experimental groups. Each rabbit received subcutaneous injections of benzene on the back every other day, followed by daily cyclophosphamide injections via the ear vein for the prescribed number of times. Blood routine examinations were performed every 6 days, and small samples of femoral bone were collected for bone marrow histopathological examination before modeling and at 36 days after modeling. **Results:** Comparison of white blood cell, erythrocyte, and platelet counts among the nine groups showed successful modeling in Groups 4–9, with daily mean reduction rates in Groups 7, 8, and 9 differing significantly from those in other groups ($P<0.05$). In Group 7, bone marrow sections showed hypoplastic marrow, reduced hematopoietic tissue, decreased or absent megakaryocytes, and increased adipocytes. Follow-up observation revealed sustained bone marrow suppression in Group 7, consistent with the clinical characteristics of the disease. **Conclusion:** A stable and efficient aplastic anemia model can be established in New Zealand rabbits using a combination of eight subcutaneous injections of benzene at 1.5 mL/kg and four intravenous injections of cyclophosphamide at 10 mg/kg.

Key words: aplastic anemia; benzene; cyclophosphamide; orthogonal test; animal models

Introduction

Aplastic anemia (AA) is a hematopoietic failure syndrome characterized by bone marrow hypoplasia, pancytopenia, and manifestations of anemia, hemorrhage,

and infection. The average annual incidence in China is 0.74 per 100,000 population, occurring across all age groups with higher rates among the elderly and no significant gender difference [1]. AA carries high mortality and is difficult to cure; acute AA has an extremely poor prognosis, with only a minority of milder cases achieving remission or cure [2]. To investigate the pathogenesis and treatment of AA, appropriate animal models are essential.

The pathogenesis of AA is extremely complex and remains inconclusive, with various modeling methods including physical, chemical, physicochemical, and immune-mediated approaches [3]. However, current methods suffer from limitations such as unstable AA phases, inconsistent reduction across the three blood cell lineages, complex procedures, and low induction rates [4-8]. To establish a more clinically relevant and stable AA animal model, our research group analyzed the advantages and disadvantages of multiple modeling methods [5-12] and identified a combined benzene and cyclophosphamide approach in New Zealand rabbits. Using experimental parameters from these methods, we conducted an orthogonal design to establish a stable AA animal model that better reflects clinical characteristics and to identify an optimal protocol.

Materials and Methods

1.1.1 Experimental Animals

Ninety healthy adult New Zealand rabbits of both sexes, weighing $2\$\pm0.2kg, were provided by the Experimental Animal Center of Chongqing Medical University [Production Licen SCXK(Yu)2012-0001]. All animals were housed under identical laboratory conditions (temperature 22±2°C, hu 12-hour light-dark cycle). All animal procedures complied with ethical standards.

1.1.2 Reagents and Instruments

Injections of benzene (analytical grade, Sinopharm Chemical Reagent Shenyang Co., Ltd.); cyclophosphamide (Jiangsu Hengrui Medicine Co., Ltd.); EDTA-2K (Beijing Solarbio Science & Technology Co., Ltd.); HEMAVET 950FS automatic five-part hematology analyzer (DREW, USA); SM2000R paraffin microtome (Leica, Germany); Olympus-BX51 optical microscope (OLYMPUS, Japan).

1.2.1 Model Establishment and Grouping

Referencing the four-factor, three-level orthogonal table L9 (3^4) in the second edition of *Medical Statistics* edited by Yan Hong [13], 90 New Zealand rabbits were randomly divided into nine experimental groups (Table 1) with 10 rabbits per group. Experimental groups were treated strictly according to the orthogonal design table: benzene was administered first via subcutaneous injection on the back every other day until the prescribed number of injections was completed; cyclophosphamide was then administered via ear vein injection daily until the prescribed number of injections was completed.

1.2.2 Index Collection and Detection

1.2.2.1 General Observation Throughout the experiment, we observed and recorded the rabbits' mental status, skin and fur condition, body weight changes, time of death, and calculated survival rates.

1.2.2.2 Blood Routine Examination Blood routine examinations were performed every 6 days. Fur near the central ear artery was removed, the area was cleaned with alcohol, and after vessel engorgement, 1 mL of blood was drawn using a 2 mL syringe and immediately transferred to EDTA dipotassium salt collection tubes. The tubes were inverted to prevent coagulation, and complete blood counts were analyzed within 2 hours using an automatic hematology analyzer to measure white blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts.

1.2.2.3 Bone Marrow Histopathological Observation Before modeling and at 36 days after modeling, rabbits were euthanized by ear vein air injection. Under sterile conditions, a small segment of femur was dissected and immediately fixed in 4% paraformaldehyde. After dehydration, paraffin embedding, sectioning, and hematoxylin-eosin staining, cellular morphology was observed under an optical microscope.

1.2.3 Criteria for Model Success

A diagnosis of aplastic anemia was established when WBC decreased by 50%, RBC and platelets decreased by 20%, and bone marrow smears or sections showed hypoplastic marrow with increased adipose tissue [14-15].

1.3 Statistical Analysis

Data were analyzed using SPSS 22.0 statistical software. All data are expressed as mean ± standard deviation. Differences in daily mean reduction rates of cell counts were analyzed using a two-level model with fixed measurement time. $P<0.05$ was considered statistically significant.

Results

2.1.1 General Observations

Six days after drug administration, some rabbits began showing sluggish responses, pale ear margins, dull fur, and partial alopecia. The time to reach diagnostic criteria and mortality rates for each group are shown in Table 2 . In Groups 1, 2, and 3, deaths occurred as early as days 12, 24, and 30 post-administration, respectively, but blood routine parameters never met diagnostic criteria, with mortality rates of 10%, 20%, and 0%. In Groups 4, 5, and 6, deaths occurred as early as days 16, 22, and 16, respectively, with blood routine parameters meeting diagnostic criteria on days 21, 21, and 24, and mortality

rates of 40% in each group. In Groups 7, 8, and 9, deaths occurred as early as days 3, 6, and 9, respectively, with blood routine parameters meeting diagnostic criteria on days 18, 18, and 30, and mortality rates of 70%, 60%, and 70%.

2.1.2 Survival Rate

Survival rates differed among the nine groups after combined benzene and cyclophosphamide treatment, decreasing with increasing benzene dose. Survival function analysis using SPSS 22.0 revealed that only benzene dose significantly affected survival rate ($P<0.05$), while cyclophosphamide dose (Figure 1B [Figure 1: see original paper]) and injection frequency showed no significant effects ($P>0.05$, Figure 1A [Figure 1: see original paper]).

2.2.1 Red Blood Cell Count

Intergroup comparison of RBC counts (Table 3) showed that Groups 7, 8, and 9 had greater daily mean reduction rates than some other groups. Specifically, Group 7 differed significantly from Groups 2, 3, 4, 5, and 6 ($P<0.05$); Group 8 differed significantly from Groups 1, 2, 3, 4, 5, and 6 ($P<0.001$) and from Group 9 ($P<0.05$); Group 9 differed significantly from Groups 2, 4, and 8 ($P<0.05$). Ranked by the number of statistically significant differences with other groups: Group 8 > Group 7 > Group 9.

2.2.2 White Blood Cell Count

Intergroup comparison of WBC counts (Table 4) showed that Groups 7, 8, and 9 had greater daily mean reduction rates than some other groups. Group 7 differed significantly from Groups 1, 2, 3, 4, 5, 6, and 8 ($P<0.01$); Group 8 differed significantly from Groups 2, 6, and 7 ($P<0.05$); Group 9 differed significantly from Groups 2, 3, and 6 ($P<0.05$). Ranked by the number of statistically significant differences: Group 7 > Group 8 = Group 9.

2.2.3 Platelet Count

Intergroup comparison of platelet counts (Table 5) showed that Groups 7, 8, and 9 had greater daily mean reduction rates than some other groups. Group 7 differed significantly from Groups 1, 2, 3, 4, 5, and 6 ($P<0.05$); Group 8 differed significantly from Groups 5 and 6 ($P<0.05$); Group 9 differed significantly from Group 6 ($P<0.05$). Ranked by the number of statistically significant differences: Group 7 > Group 8 > Group 9.

Dynamic changes in RBC, WBC, and platelet counts are shown in Figure 2 [Figure 2: see original paper]. For RBC percentage change relative to day 0 (Figure 2A), Group 8 showed the greatest decline but continued to decrease substantially at days 18 and 24, indicating poor model stability. Group 7 showed rapid and sustained decline. For WBC percentage change (Figure 2B), Groups 6, 8,

and 9 showed substantial increases after day 18, which was unfavorable for modeling; only Group 7 showed sustained decline. For platelet percentage change (Figure 2C), Groups 8 and 9 showed obvious recovery at days 12 and 18 before declining again at day 24; Group 7 was the only group showing consistently stable decline. Thus, Group 7 was the optimal group among the nine, meeting diagnostic criteria with the most stable decline.

2.2.4 Bone Marrow Histological Changes

Comparison of bone marrow pathology sections (Figure 3 [Figure 3: see original paper]) revealed that Groups 1, 2, and 3 showed abundant hematopoietic tissue with intact structure, not meeting AA criteria. Groups 4–9 showed hypoplastic marrow, reduced hematopoietic tissue, decreased or absent megakaryocytes, and increased adipocytes, consistent with AA characteristics.

Based on these results, Groups 4–9 met all AA criteria, with Groups 7, 8, and 9 showing statistically significant differences in daily mean reduction rates across all three lineages compared to other groups, particularly Group 7.

Discussion

Animal model establishment is crucial for studying toxic diseases, with various methods offering distinct advantages and disadvantages that can be selected based on experimental objectives [16]. Reviewing domestic and international AA modeling methods [5-11] reveals that most AA models use mice, which can be successfully established. However, dose control is challenging: excessive doses cause high mortality, while insufficient doses fail to achieve consistent suppression. Additionally, mice have limited blood volume (5.85 mL/100 g whole blood, 3.15 mL/100 g plasma) [17-18], making repeated blood sampling difficult without affecting the hematological system. Considering these limitations, our group selected New Zealand rabbits, which allow repeated blood sampling with minimal impact, facilitating model establishment and stable monitoring [19-20].

Benzene is a commonly used chemical toxicant that accumulates in bone marrow during chronic poisoning, causing hematological damage [4]. Velasco et al. [5] established AA models in CD1 mice by injecting 2 mL/kg benzene 3–5 times weekly for 20 doses, resulting in reduced peripheral blood and bone marrow cell counts. Aerosolized benzene for 10 weeks induced AA in Kunming mice, primarily manifested by impaired erythroid colony formation and reduced erythroid progenitors [6], but this method is complex with low induction rates. Zhang et al. [7] first established an immunosuppressed animal model using a large cyclophosphamide dose of 200 mg/kg via tail vein injection followed by 7 days of maintenance dosing at 30 mg/kg intraperitoneally, achieving significant peripheral cytopenia and bone marrow suppression. However, cyclophosphamide alone causes significant leukocyte reduction while erythrocyte decline is slow and minimal, with rapid hematological recovery after drug cessation, indicating reversible bone marrow injury [8]. Sun et al. [9] obtained AA models using

^{60}Co-γ irradiation, cyclophosphamide, and chloramphenicol, but this required specialized equipment and personnel, was inconvenient to operate, and involved difficult radiation dose control [3]. Our study adopted a scientifically rational combined drug approach based on literature and previous experience.

The results demonstrate successful establishment of New Zealand rabbit AA models. Among experimental groups, Groups 1, 2, and 3 failed; Groups 4, 5, and 6 successfully established models but showed slow, inconsistent declines across three lineages with long cycles and poor stability. Groups 7, 8, and 9 met all AA criteria, with Group 7 being optimal, showing marked changes in general condition, three-lineage blood counts, and bone marrow histology, indicating an ideal drug dosage for AA modeling. Blood routine examination on day 51 in surviving Group 7 rabbits confirmed persistent AA diagnostic criteria, demonstrating strong model stability. Compared with single-agent approaches characterized by long administration times and inconsistent AA onset, our combined benzene and cyclophosphamide regimen offers shorter modeling cycles, simpler operation, higher success rates, more fixed timing of bone marrow dysfunction onset, and parameters similar to human AA.

AA pathogenesis research has progressed through three stages. Stage 1 attributed AA primarily to drug and chemical toxicity. Stage 2, with advances in microscopic sciences, revealed high pathological heterogeneity, with "seed" (hematopoietic stem cell abnormalities), "soil" (hematopoietic microenvironment abnormalities), and "bug" (immune function abnormalities) theories becoming mainstream. Stage 3 has seen increasing acceptance of T-cell hyperfunction-induced bone marrow damage [22-24], confirming that AA pathogenesis is complex and not fully understood, primarily involving immune dysfunction [25-27]. Our study employed Stage 1 mechanisms using drug and chemical toxicity to induce AA. Group 7 also met AA characteristics and diagnostic criteria—primarily peripheral pancytopenia and bone marrow failure—though further mechanistic studies are needed.

This study has limitations. Benzene, one of the earliest agents for AA modeling, can cause hematopoietic dysfunction in multiple mouse strains [28]. Group 7 showed early mortality and relatively high death rates, primarily due to benzene dosage, which is indispensable for AA modeling. While successful, excessive mortality increases experimental costs. Future studies should optimize benzene dosage to make this modeling method more suitable for domestic researchers investigating AA.

In summary, a combination of benzene at 1.5 mL/kg for 8 doses and cyclophosphamide at 10 mg/kg for 4 doses establishes a New Zealand rabbit AA model with short modeling cycles, clinical AA characteristics, and stable performance, representing a widely applicable method for animal experiments.

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Note: Figure translations are in progress. See original paper for figures.